THE PREPROGLUCAGON DERIVED
PEPTIDES AND ENERGY HOMEOSTASIS

A thesis submitted for the degree of
Doctor of Philosophy

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Abstract

Obesity is a major contributor to the development of chronic diseases, and there is a paucity of effective treatments. Recent studies suggest that co-agonists at the glucagon-like peptide-1 (GLP-1) and glucagon receptor efficaciously reduce body weight and improve glucose homeostasis. This thesis explores the effects of glucagon, GLP-1 and the endogenous GLP-1/glucagon receptor co-agonist oxyntomodulin, on appetite and glucose homeostasis and their mechanisms.

As expected, peripheral injection of GLP-1 or glucagon to fasted mice transiently reduced food intake. Interestingly, subanorectic doses of GLP-1 and glucagon potently inhibited food intake when combined. Agonists at the GLP-1 (GLPAg) and glucagon (GCGAg) receptors designed in this laboratory were found to have receptor affinities comparable with those of GLP-1 and GCG, but with a prolonged duration of action. When administered chronically, individually and in combination, to an obese mouse model, the combination of these peptides appeared to cause superior reduction in body weight and improvement in glucose tolerance compared to the individual peptides.

The receptors and central appetite regulating centres involved in the response to anorectic doses of GLP-1, glucagon and oxyntomodulin were investigated. The pattern of c-fos immunoreactivity in response to glucagon was examined for the first time and appeared indistinguishable from that induced by GLP-1. Oxyntomodulin appeared to induce greater c-fos activation in the nucleus tractus solitarius (NTS) than either glucagon or GLP-1 at equivalently anorectic doses. No difference in the activation of catecholaminergic, preproglucagon or POMC expressing neurons in the NTS was seen between the three peptides. The anorectic effects of both oxyntomodulin and glucagon appeared to depend on the presence of the GLP-1 receptor.

The effects of both glucagon and oxyntomodulin on blood glucose and insulin release in mice were investigated and found to be highly dose dependant. Surprisingly, high doses of glucagon did not have a detectable hyperglycemic effect and some evidence suggested this may be due to cross reactivity with the GLP-1 receptor. The GLP-1 receptor antagonist EX 9-39 had an unexpected hyperglycemic effect, present in GLP-1 receptor knockout mice suggesting an alternative mechanism of action.

Overall the work contained in this thesis has added mechanistic information to our knowledge about the anorectic and glucoregulatory effects of the preproglucagon derived peptides and supports development of synthetic agonists of the glucagon and GLP-1 receptors as treatments for obesity.
Declaration of contributors

The author performed the majority of the work contained within this thesis. All collaboration and assistance is described below:

**Experimental chapter 1:**

Receptor Binding Assays for the screening of analogues were done in collaboration with James Plumer, Klara Hostomska, James Minnion and Joyceline Cuenco-Shillito.

Feeding studies, pair-feeding studies, pharmacokinetic studies and glucose tolerance testing were carried out with the help of the ‘G series’ team, in particular James Minnion, Joyceline Cuenco-Shillito, James Plumer, Katherine McCullough, Sam Price and Tanya Stezhka.

**Experimental chapter 2:**

Dose response and combinatorial c-fos studies were carried out in collaboration with Katherine McCullough. The comparative glucagon, GLP-1, oxyntomodulin c-fos study was carried out with the assistance of John Tadross under whose guidance the dual immunohistochemistry was also carried out.

GLP-1R knockout mice were bred with the kind permission of Dr Daniel Drucker (Samuel Lunenfeld Research Institute, University of Toronto).

**Experimental chapter 3:**

Studies measuring blood glucose carried out with the assistance of Joyceline Cuenco-Shillito, Sam Price, Jamie Plumer and in particular Tanya Stezhka.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>Avidin-biotin complex</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetyl choline</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti-related protein</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>Area postrema</td>
</tr>
<tr>
<td>ARC</td>
<td>Arcuate nucleus of the hypothalamus</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CA</td>
<td>catecholamine</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CART</td>
<td>Cocaine-and amphetamine-regulated transcript protein</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CeA</td>
<td>Central nucleus of the Amygdala</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotrophin-releasing hormone</td>
</tr>
<tr>
<td>DAB</td>
<td>3’3’-diaminobenzidine tetrahydrochloride hydrate</td>
</tr>
<tr>
<td>DMN</td>
<td>Dorsomedial nucleus of the hypothalamus</td>
</tr>
<tr>
<td>DMV</td>
<td>Dorsal motor nucleus of the vagus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPP-IV</td>
<td>Dipeptidyl peptidase-IV</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-related kinase</td>
</tr>
<tr>
<td>EX-4</td>
<td>Exendin-4</td>
</tr>
<tr>
<td>EX 9-39</td>
<td>Exendin 9-39</td>
</tr>
<tr>
<td>FFA</td>
<td>Free Fatty Acid</td>
</tr>
<tr>
<td>FI</td>
<td>Food intake</td>
</tr>
<tr>
<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GCGR</td>
<td>Glucagon receptor</td>
</tr>
<tr>
<td>GHRH</td>
<td>Growth hormone releasing hormone</td>
</tr>
<tr>
<td>GHSR</td>
<td>Growth hormone secretagogue receptor</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
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<td>GLP-2</td>
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</tr>
<tr>
<td>GLP-1R</td>
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</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GRP</td>
<td>Gastrin releasing peptide</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association studies</td>
</tr>
<tr>
<td>GTT</td>
<td>Glucose tolerance test</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>ICV</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LHA</td>
<td>Lateral hypothalamic area of the hypothalamus</td>
</tr>
<tr>
<td>MC4R</td>
<td>Melanocortin 4 receptor</td>
</tr>
<tr>
<td>MCH</td>
<td>Melanocortin-concentrating hormone</td>
</tr>
<tr>
<td>ME</td>
<td>Median eminence</td>
</tr>
<tr>
<td>MEMRI</td>
<td>Manganese enhanced magnetic resonance imaging</td>
</tr>
<tr>
<td>MFB</td>
<td>Medial forebrain bundle</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSH</td>
<td>Melanocyte-stimulating hormone</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
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<tr>
<td>NTS</td>
<td>Nucleus of the tractus solitarius</td>
</tr>
<tr>
<td>OXM</td>
<td>Oxyntomodulin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PACAP</td>
<td>Pituitary adenylate cyclase-activating poly-peptide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBN</td>
<td>Parabrachial nucleus</td>
</tr>
<tr>
<td>PC</td>
<td>Prohormone convertase</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>POMC</td>
<td>Proopiomelanocortin</td>
</tr>
<tr>
<td>PP</td>
<td>Pancreatic polypeptide</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus of the hypothalamus</td>
</tr>
<tr>
<td>PYY</td>
<td>Peptide YY</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>s/c</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SCN</td>
<td>Suprachiasmatic nucleus of the hypothalamus</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SON</td>
<td>Supraoptic nucleus of the hypothalamus</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
<tr>
<td>VMN</td>
<td>Ventromedial nucleus of the hypothalamus</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
Table of Contents

Abstract .................................................................................................................................................. 2

Declaration of contributors .................................................................................................................. 3

Acknowledgements .............................................................................................................................. 4

Abbreviations ........................................................................................................................................ 5

Table of figures ...................................................................................................................................... 12

List of tables .......................................................................................................................................... 15

1 GENERAL INTRODUCTION ................................................................................................................. 16

1.1 Introduction ..................................................................................................................................... 17

1.2 Energy Homeostasis ....................................................................................................................... 18

1.3 Overview of appetite control systems ........................................................................................... 21

1.3.1 Neurocircuity ............................................................................................................................... 21

1.3.1.1 The Hypothalamus .................................................................................................................. 23

1.3.1.2 Brainstem appetite regulating centres ..................................................................................... 28

1.3.1.3 Regulation of appetite by reward centres ............................................................................... 30

1.3.2 Gut-Brain signalling ................................................................................................................... 32

1.3.2.1 Nutrient sensing ...................................................................................................................... 32

1.3.2.2 Nervous communication between the gut and brain ............................................................. 32

1.3.2.3 Gut Hormones ......................................................................................................................... 33

1.4 The Preproglucagon Derived Peptides ....................................................................................... 35

1.4.1 The Preproglucagon Gene .......................................................................................................... 35

1.4.2 Glucagon ..................................................................................................................................... 37

1.4.2.1 Peptide .................................................................................................................................. 37

1.4.2.2 Stimuli for Glucagon secretion .............................................................................................. 37

1.4.2.3 The Glucagon Receptor ......................................................................................................... 38

1.4.2.4 Physiological Roles of Glucagon ........................................................................................... 38

1.4.3 GLP-1 .......................................................................................................................................... 42

1.4.3.1 Peptide .................................................................................................................................. 42

1.4.3.2 Stimulation of GLP-1 secretion ............................................................................................. 42

1.4.3.3 The GLP-1 Receptor .............................................................................................................. 43

1.4.3.4 Physiological roles of GLP-1 ............................................................................................... 43

1.4.4 Oxyntomodulin .......................................................................................................................... 45

1.4.4.1 Peptide .................................................................................................................................. 45

1.4.4.2 Receptors for Oxyntomodulin ............................................................................................... 45

1.4.4.3 Physiological Roles of Oxyntomodulin ............................................................................... 46

1.5 Overview of thesis .......................................................................................................................... 48

2 EXPERIMENTAL CHAPTER ONE ........................................................................................................ 49
2.1 Pharmacotherapies for obesity ........................................................................50
  2.1.1 GLP-1 receptor agonists as treatments for obesity ...........................................53
  2.1.1.1 GLP-1/glucagon receptor co-agonists ..........................................................56
  2.1.2 Our strategy .....................................................................................................58

2.2 Chapter Summary and Aims ..........................................................................59

2.3 Materials and Methods .................................................................................60
  2.3.1 Receptor Binding Assays (RBA) .....................................................................60
  2.3.1.1 Radioligand production ................................................................................60
  2.3.1.2 Preparation of membrane from cell lines expressing GLP-1 R or GCGR .........60
  2.3.1.3 Preparation of membrane from mouse and rat tissue ....................................61
  2.3.1.4 Receptor Binding Assay ................................................................................61
  2.3.2 In vivo studies ................................................................................................62
  2.3.2.1 Animals .........................................................................................................62
  2.3.2.2 Peptides .........................................................................................................62
  2.3.2.3 Acute Feeding studies ....................................................................................62
  2.3.2.4 Chronic feeding study in Diet Induced Obese (DIO) mice .........................63
  2.3.2.5 Pair-feeding Studies .....................................................................................65
  2.3.2.6 Pharmacokinetic studies .............................................................................66
  2.3.3 Statistics .........................................................................................................68

2.4 Results .............................................................................................................69
  2.4.1 Receptor Binding .............................................................................................69
  2.4.1.1 Glucagon .......................................................................................................69
  2.4.1.2 GLP-1 ...........................................................................................................69
  2.4.1.3 Exendin 4 (EX-4) .........................................................................................71
  2.4.1.4 GLPAG: A GLP-1 receptor agonist ...............................................................71
  2.4.1.5 GCGAg: A glucagon receptor agonist .........................................................71
  2.4.2 Acute effects on food intake ...........................................................................73
  2.4.2.1 Food intake in response to a range of glucagon doses .....................................73
  2.4.2.2 Food intake in response to a range of GLP-1 doses .......................................74
  2.4.2.3 Acute food intake in response to GLPAG ...................................................75
  2.4.2.4 Acute food intake in response to GCGAg ...................................................77
  2.4.2.5 The effects of glucagon and GLP-1 individually and in combination ...........79
  2.4.3 Pharmacokinetic studies in rats ......................................................................81
  2.4.3.1 GLPAG .......................................................................................................81
  2.4.3.2 GCGAg .......................................................................................................82
  2.4.4 Effect of chronic peptide administration on food intake and body weight in rats .................................................................................................................83
  2.4.4.1 EX-4 ..........................................................................................................83
  2.4.4.2 GCGAg .......................................................................................................85
  2.4.5 Effect of chronic administration of GLPAG and GCGAg alone and in combination in DIO mice ........................................87
  2.4.5.1 Food intake and body weight ........................................................................87
  2.4.5.2 Intrapertoneal glucose tolerance test .........................................................90

2.5 Discussion ...........................................................................................................92
  2.5.1 Action of glucagon ..........................................................................................92
  2.5.1.1 Which receptor does glucagon act at to inhibit food intake? ...................94
  2.5.2 Effect of combined administration of GLP-1 and Glucagon .........................94
  2.5.3 Action of GLPAG ............................................................................................95
3 EXPERIMENTAL CHAPTER TWO .................................................. 103

3.1 Introduction .............................................................................. 104

3.1.1 The hormonal regulation of appetite .................................. 104
3.1.2 Anorectic mechanisms of GLP-1 ........................................ 104
3.1.3 Anorectic mechanisms of Glucagon ................................. 105
3.1.4 Anorectic mechanisms of Oxyntomodulin ...................... 107
3.1.5 The brainstem and central nucleus of the amygdala .......... 108
  3.1.5.1 The Nucleus Tractus Solitarius ................................ 108
  3.1.5.2 NTS neuronal populations ........................................ 111
  3.1.5.3 The parabrachial nucleus ........................................ 116
  3.1.5.4 The central nucleus of the amygdala ....................... 116
3.1.6 Background on experimental techniques and models used in this chapter ......................................................... 117
  3.1.6.1 Immunohistochemistry .......................................... 117
  3.1.6.2 c-fos .................................................................. 120
  3.1.6.3 Models of an absence of GLP-1 receptor signalling .... 124
3.1.7 Chapter Summary and Aims .................................................. 128

3.2 Materials and Methods ............................................................ 129

3.2.1 Animals .............................................................................. 129
  3.2.1.1 GLP-1 Receptor Knockout mice .................................. 129
3.2.2 Acute Feeding studies ......................................................... 129
  3.2.2.1 Procedure (excluding studies in GLP-1R knockout mice) .......................................................... 129
  3.2.2.2 Procedure (GLP-1R knockout mice and wild type littermates) ................................................. 130
3.2.3 c-fos immunohistochemistry studies in mice ..................... 130
  3.2.3.1 Animal conditions and tissue preparation .................... 131
  3.2.3.2 c-fos immunohistochemistry .................................... 131
  3.2.3.3 Analysis ................................................................. 132
3.2.4 Dual Immunohistochemistry for c-fos and markers of neuronal populations in the NTS .................................................. 133
3.2.5 Statistical analysis ............................................................... 134

3.3 Results .................................................................................... 135

3.3.1 Effect of GLP-1 receptor antagonist on the anorectic effect of glucagon .......................................................... 135
3.3.2 Effect of a glucagon receptor antagonist on the anorectic effect of glucagon .................................................. 135
3.3.3 Effect of EX-4, PYY₃-₃₆, glucagon (GCG) and oxyntomodulin (OXM) on food intake in GLP-1R knockout mice 137
3.3.4 The effect of anorectic doses of glucagon on c-fos immunoreactivity .......................................................... 139
3.3.5 The effect of anorectic doses of GLP-1 on c-fos immunoreactivity .......................................................... 143
3.3.6 The effect of combined administration of GLP-1 and glucagon on c-fos immunoreactivity ................................ 145
3.3.7 Comparison of effects of GLP-1, glucagon and OXM on c-fos expression .......................................................... 148
3.3.7.1 Dose finding .................................................................................. 148
3.3.7.2 Comparison of effects of GLP-1, glucagon and OXM on c-fos expression .................................................. 149
3.3.8 Investigation of neuronal populations in the NTS activated by GLP-1, glucagon and oxyntomodulin 152
3.3.8.1 TH expressing neurons .................................................................. 152
3.3.8.2 ACTH expressing neurons ................................................................. 156
3.3.8.3 GLP-1 expressing neurons ................................................................. 158

3.4 Discussion .............................................................................................. 159
3.4.1 The action of glucagon ..................................................................... 159
3.4.2 The activation of central appetite regulating centres by glucagon ............ 160
3.4.3 The action of GLP-1 and a comparison to that of glucagon .................... 163
3.4.4 The action of combined GLP-1 and glucagon ....................................... 164
3.4.5 Comparison of effects of GLP-1, glucagon and OXM on c-fos expression ........ 165
3.4.5.1 Investigation of neuronal populations in the NTS activated by glucagon, GLP-1 and oxyntomodulin ...................................................... 168
3.4.6 Discussion of methodologies used and their limitations ....................... 170
3.4.6.1 Pharmacological blockade and genetic deletion of the GLP-1R ........... 170
3.4.6.2 Dual immunohistochemistry for identification of c-fos expressing neurons .......... 170
3.4.7 Conclusions and future directions .................................................... 172

4 EXPERIMENTAL CHAPTER THREE ................................................................ 173

4.1 Introduction ............................................................................................ 174
4.1.1 Glucose ......................................................................................... 174
4.1.2 Glucose homeostasis ...................................................................... 174
4.1.2.1 Glucose sensing ........................................................................ 174
4.1.2.2 Glucose stores .......................................................................... 178
4.1.2.3 Endocrine Regulation of Blood Glucose ........................................ 178
4.1.2.4 Oxyntomodulin and glucose homeostasis ..................................... 184
4.1.3 Chapter Summary and Aims .............................................................. 185

4.2 Materials and Methods ......................................................................... 186
4.2.1 Measurement of the glucose and insulin response to peripherally administered peptides .......... 186
4.2.1.1 Animals ................................................................................ 186
4.2.1.2 Study procedure .................................................................... 186
4.2.1.3 Studies in GLP-1R knockout mice .............................................. 187
4.2.2 Measurement of liver glycogen .......................................................... 188
4.2.3 Statistical Analysis .......................................................................... 189

4.3 Results ...................................................................................................... 190
4.3.1 The effects of oxyntomodulin on glucose and insulin release in wild type mice .................................................................................. 190
4.3.2 The effects of a range of doses of glucagon on glucose and insulin release in wild type mice .... 193
4.3.3 The effects of a range of low doses of glucagon on glucose and insulin release in wild type mice .196
4.3.4 Effects of glucagon and oxyntomodulin on blood glucose and insulin .......................................................... 199
4.3.5 The effect of a high dose of glucagon on glucose release shortly after injection in wild type mice. 200
4.3.6 The effect of low and high dose glucagon on liver glycogen levels ......................................................... 202
4.3.7 The effect of high and low dose glucagon on blood glucose and insulin release in wild type and GLP-1R knockout mice .................................................................................. 203
4.3.8 The effect of high and low dose oxyntomodulin on blood glucose and insulin release in wild type and GLP-1R knockout mice ................................................................. 207
4.3.9 The effect of high and low dose glucagon on blood glucose in the presence and absence of EX 9-39 .............................................................. 211
4.3.10 The effects of a range of doses of EX 9-39 on glucose in fed wild type mice ........................................ 213
4.3.11 The effect of EX 9-39 on blood glucose and insulin release in wild type and GLP-1R knockout mice .......................................................... 215

4.4 Discussion ........................................................................................................................................... 218

4.4.1 Oxyntomodulin ................................................................................................................................. 218
   4.4.1.1 The effects of oxyntomodulin on blood glucose and plasma insulin in wild type mice ........ 218
   4.4.1.2 The effect of oxyntomodulin on blood glucose and insulin release in GLP-1R knockout mice 219
4.4.2 Glucagon ........................................................................................................................................... 219
   4.4.2.1 The effects of glucagon on blood glucose and insulin release ............................................. 219
   4.4.2.2 The effect of glucagon on blood glucose and insulin release in GLP-1R knockout mice ........ 220
4.4.3 The effects of EX 9-39 on blood glucose and insulin release ......................................................... 221
4.4.4 Discussion of methodologies used and their limitations ................................................................. 222
   4.4.4.1 Investigations of glucose and insulin release in wild type mice ........................................ 222
   4.4.4.2 Investigations of glucose and insulin release in GLP-1R knockout mice ............................. 223
4.4.5 Conclusions and Future Directions ............................................................................................... 223

5 GENERAL DISCUSSION .......................................................................................................................... 225

6 APPENDICES .......................................................................................................................................... 233

6.1 Appendix One: radioimmunoassays ................................................................................................. 234

6.2 Appendix Two: The effect of gcgAg on acute food intake in rats .................................................. 236

6.3 Appendix Three: The Effect of GLP-1 on food intake in the presence and absence of EX 9-39 ........ 237

6.4 Appendix Four: Coordinates of sections used for c-fos immunohistochemistry ............................. 238

6.5 Appendix Five: ACTH immunoreactivity in the hypothalamus ....................................................... 239

6.6 Appendix Six: Solutions ....................................................................................................................... 240
## Table of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Overview of appetite control systems</td>
<td>22</td>
</tr>
<tr>
<td>1.2</td>
<td>Diagrammatic representation of the hypothalamus</td>
<td>24</td>
</tr>
<tr>
<td>1.3</td>
<td>Proglucagon processing in the pancreas and intestinal tract</td>
<td>35</td>
</tr>
<tr>
<td>1.4</td>
<td>Summary of interaction between glucagon and the HPA axis.</td>
<td>41</td>
</tr>
<tr>
<td>2.1</td>
<td>Amino acid structures of GLP-1, and the GLP-1R agonists EX-4 and Liraglutide</td>
<td>55</td>
</tr>
<tr>
<td>2.2</td>
<td>Competitive binding of $^{125}$I-glucagon and glucagon at the human, mouse, and rat glucagon receptors</td>
<td>70</td>
</tr>
<tr>
<td>2.3</td>
<td>Competitive binding of $^{125}$I-GLP-1 and GLP-1 at the human, mouse, and rat GLP-1Rs</td>
<td>70</td>
</tr>
<tr>
<td>2.4</td>
<td>A Competitive binding of $^{125}$I-GLP-1 and EX-4 or GLPAg at the human, mouse, and rat GLP-1Rs</td>
<td>72</td>
</tr>
<tr>
<td>2.5</td>
<td>A Competitive binding of $^{125}$I-glucagon and GCGAg at the human, mouse, and rat glucagon receptors</td>
<td>72</td>
</tr>
<tr>
<td>2.6</td>
<td>Effect of a range of doses of glucagon on food intake</td>
<td>73</td>
</tr>
<tr>
<td>2.7</td>
<td>Effect of a range of GLP-1 doses on food intake in fasted mice.</td>
<td>74</td>
</tr>
<tr>
<td>2.8</td>
<td>Effect of GLPAg on food intake over 24 hours</td>
<td>76</td>
</tr>
<tr>
<td>2.9</td>
<td>Effect of GCGAg on food intake over 24 hours</td>
<td>78</td>
</tr>
<tr>
<td>2.10</td>
<td>Effect of GLP-1 and glucagon at a range of doses and in combination on food intake</td>
<td>80</td>
</tr>
<tr>
<td>2.11</td>
<td>Pharmacokinetics of subcutaneously administered GLPAg in rats</td>
<td>81</td>
</tr>
<tr>
<td>2.12</td>
<td>Pharmacokinetics of subcutaneously administered GCGAg in rats</td>
<td>82</td>
</tr>
<tr>
<td>2.13</td>
<td>Effect of daily EX-4 for 7 days on food intake and body weight in rats</td>
<td>84</td>
</tr>
<tr>
<td>2.14</td>
<td>Effect of daily GCGAg for 7 days on food intake and body weight in rats</td>
<td>86</td>
</tr>
<tr>
<td>2.15</td>
<td>Effect of daily GLPAg, GCGAg or GLPAg+GCGAg for 70 days on food intake in diet induced obese mice</td>
<td>88</td>
</tr>
<tr>
<td>2.16</td>
<td>Effect of daily GLPAg, GCGAg or GLPAg+GCGAg for 70 days on body weight in diet induced obese mice</td>
<td>89</td>
</tr>
<tr>
<td>2.17</td>
<td>Effect of daily GLPAg, GCGAg or GLPAg+GCGAg for 70 days on response to glucose tolerance test</td>
<td>91</td>
</tr>
<tr>
<td>3.1</td>
<td>Schematic showing subdivisions of the NTS and the names by which they are described in this thesis</td>
<td>109</td>
</tr>
<tr>
<td>3.2</td>
<td>Four immunohistochemically distinct neuronal populations in the caudal NTS involved in appetite regulation</td>
<td>111</td>
</tr>
<tr>
<td>3.3</td>
<td>Preproglucagon neurons in the mouse brainstem</td>
<td>114</td>
</tr>
<tr>
<td>3.4</td>
<td>Key features of the c-fos gene</td>
<td>121</td>
</tr>
<tr>
<td>3.5</td>
<td>Effect of GLP-1 and glucagon receptor antagonists on the anorectic effect of glucagon</td>
<td>136</td>
</tr>
<tr>
<td>3.6</td>
<td>Effect of EX-4, PYY3-36, glucagon and oxyntomodulin on food intake in GLP-1R knockout mice</td>
<td>138</td>
</tr>
<tr>
<td>3.7</td>
<td>The effects of glucagon on food intake and c-fos immunoreactivity in fasted mice</td>
<td>140</td>
</tr>
<tr>
<td>3.8</td>
<td>Representative photomicrographs of c-fos immunoreactivity 90 minutes</td>
<td>141</td>
</tr>
<tr>
<td>3.9</td>
<td>The effects of GLP-1 on food intake and c-fos immunoreactivity in fasted mice</td>
<td>144</td>
</tr>
<tr>
<td>3.10</td>
<td>The effects of coadministration of GLP-1 and glucagon on food intake and c-fos immunoreactivity in fasted mice</td>
<td>146</td>
</tr>
</tbody>
</table>
Figure 3.11: Representative photomicrographs of c-fos immunoreactivity 90 minutes after subcutaneous administration of saline, GLP-1, glucagon or GLP-1 + glucagon. .................................147
Figure 3.12: Dose finding for comparison of effects of GLP-1, glucagon and OXM on c-fos expression. .................................................................................................................148
Figure 3.13: Comparison of effects of GLP-1, glucagon and OXM on c-fos expression. ..................150
Figure 3.14: Representative photomicrographs of immunohistochemical staining for c-fos and TH in the caudal NTS. ........................................................................................................153
Figure 3.15: Representative photomicrographs of immunohistochemical staining for c-fos and TH in the caudal NTS. ........................................................................................................154
Figure 3.16: Representative photomicrographs of immunohistochemical staining for c-fos and TH in the subpostremal NTS. ........................................................................................................154
Figure 3.18: Representative photomicrographs of immunohistochemical staining for c-fos and ACTH in the caudal NTS. ........................................................................................................156
Figure 3.19: Representative photomicrographs of immunohistochemical staining for c-fos and ACTH in the subpostremal NTS. ........................................................................................................157
Figure 3.20: Representative photomicrographs of immunohistochemical staining for c-fos and GLP-1 in the caudal NTS. ........................................................................................................158
Figure 4.1: Glucose sensing in the pancreatic β-cell. .........................................................................176
Figure 4.2: Glucagon receptor signalling to increase cellular glucose output. ...............................183
Figure 4.3: Outline of in vivo study procedure when investigating the effects of glucagon or oxyntomodulin on blood glucose. ........................................................................................................187
Figure 4.4: The effect of a range of doses of oxyntomodulin on blood glucose in wild type fed mice........................................................................................................................................191
Figure 4.5: The effect of a range of doses of oxyntomodulin on plasma insulin in fed mice. ............192
Figure 4.6: The effect of a range of doses of glucagon on blood glucose in fed mice. ......................194
Figure 4.7: The effect of a range of doses of glucagon on plasma insulin in fed mice. ....................195
Figure 4.8: The effect of a range of doses of glucagon on blood glucose in fed mice. ....................197
Figure 4.9: The effect of a range of doses of glucagon on plasma insulin in fed mice. ....................198
Figure 4.10: The effect of a range of doses of glucagon on plasma glucose and insulin in fed mice. 199
Figure 4.11: The effect of a range of doses of oxyntomodulin on plasma glucose and insulin in fed mice........................................................................................................................................199
Figure 4.12: The effect of high dose glucagon blood glucose in fed mice........................................201
Figure 4.13: The effect of high and low dose glucagon on liver glycogen content in fed mice. .......202
Figure 4.14: The effect of high and low dose glucagon on blood glucose in wild type and GLP-1R knockout mice........................................................................................................................................205
Figure 4.15: The effect of high and low dose glucagon on plasma insulin in wild type and GLP-1R knockout mice........................................................................................................................................206
Figure 4.16: The effect of high and low dose oxyntomodulin on blood glucose in wild type and GLP-1R knockout mice........................................................................................................................................209
Figure 4.17: The effect of high and low dose of oxyntomodulin on plasma insulin in wild type and GLP-1R knockout mice. ........................................................................................................209
Figure 4.18: The effect of high and low dose glucagon on blood glucose in mice in the presence and absence of a GLP-1R antagonist........................................................................................................210
Figure 4.19: The effect of a range of doses of the GLP-1R antagonist EX 9-39 on blood glucose in fed mice........................................................................................................................................214
Figure 4.20: The effect of the GLP-1R antagonist EX 9-39 on blood glucose in wild type and GLP-1R knockout mice ......................................................... 216
Figure 4.21: The effect of the GLP-1R antagonist EX 9-39 on plasma insulin in wild type and GLP-1R knockout mice ................................................................. 217
Figure 6.1: Effect of GCGAg on food intake over 24 hours in rats ......................................................................................... 236
Figure 6.2: Effect of GLP-1 on food intake in the presence and absence of EX 9-39. 1 ............................................. 237
Figure 6.3: ACTH immunoreactivity in the hypothalamus .............................................................................................................. 239
List of Tables

Table 1.1: Summary of characteristics of gut hormones affecting appetite. .................................................. 34
Table 2.1: Summary of published studies where glucagon was administered to rats and acute food intake measured. ......................................................................................................................................................... 92
Table 3.1: c-fos in the hypothalamus in response to a range of doses of glucagon. ................................. 142
Table 3.2: c-fos in the hypothalamus in response to a range of doses of GLP-1................................. 142
Table 3.3: c-fos in the hypothalamus in response to GLP-1, glucagon or OXM................................. 151
Table 6.1: Coordinates of sections used for c-fos immunohistochemistry ............................................. 238
1 General Introduction
1.1 INTRODUCTION

Forty years ago obesity affected less than 3% of the adult population of the UK (Wadsworth et al. 2006). Current figures suggest 26% of adults living in the UK are classified as clinically obese (BMI ≥ 30) (Office for National Statistics, 2012). This dramatic increase in the prevalence of obesity is associated with increased all-cause mortality in obese and overweight individuals (Berrington de et al., 2010). Risks of hypertension, coronary heart disease, type 2 diabetes, certain cancers, dyslipidemia, obstructive sleep apnoea, gallstones and arthritis all increase in the obese (World Health Organisation, 2000). In addition overweight and obese individuals can also have impaired fertility and there is an increased incidence of fetal defects when the mother is obese (World Health Organisation, 2000).

Obesity was once considered to be a concern primarily in the western world, but it is now a global problem. Obesity levels have reached over 35% in Saudi Arabia, 30% in Egypt, 15% in Iran, 21% in South Africa, 15% in Zimbabwe, 22% in Chile and 16% in Malaysia (World Health Organisation, 2008). The growing scale of the obesity epidemic is likely to render gastric surgery, currently the only consistently effective long term treatment for obesity, too expensive and impractical a solution (NIH Technology Assessment Conference Panel, 1993). There is thus an urgent need for an efficacious pharmacological treatment. Understanding of the physiological systems regulating energy homeostasis is fundamental to establishing effective therapies for this worldwide health crisis. The work in this thesis aims to further our understanding of the anorectic and antidiabetic effects of the preproglucagon derived peptides glucagon, glucagon-like peptide (GLP-1) and oxyntomodulin. This class of peptides is currently being targeted for development as anti-obesity therapeutics and the first chapter of this thesis will focus on some examples of analogues of these hormones and their effects in pre-clinical rodent models.
1.2 **Energy Homeostasis**

Between the ages of 25 and 54 the average man gains 4.5 kg and the average woman 7.3 kg of body weight (Kuczmarski, 1992). An imbalance between food intake and energy expenditure of 1%, or 20 kcal a day, would lead to a 30 kg weight gain in the same period (Shin et al., 2009). In humans there is a large variation in day-to-day energy intake (Balogh et al., 1971), which is thought to reflect seasonal and social effects amongst others (de Castro, 2000). The maintenance of a relatively stable body weight under these circumstances of great variation suggests that body weight is under some degree of homeostatic regulation. Both energy intake and energy expenditure appear to be adjusted as part of this homeostatic system (Mayer et al., 1954; Janowitz and Hollander, 1955; Fregly et al., 1957; Keesey and Corbett, 1990).

1.2.1 Energy expenditure

The body uses energy for processes which can be divided into three broad categories. Basal metabolism is the energy used when an animal is at rest. Adaptive thermogenesis includes shivering and non-shivering thermogenesis and can be altered in response to fluctuations in ambient temperature, and perhaps also to changes in energy intake (DeHaven et al., 1980; Dauncey, 1981; Wijers et al., 2010). Activity related energy expenditure is divided into volitional exercise and non-exercise activity thermogenesis (Levine, 2007). Activity related thermogenesis rises and falls with increases or decreases in body weight respectively (Leibel et al., 1995).

1.2.2 Food intake

Twenty four-hour food intake is determined by the size and frequency of meals ingested. Meal size is determined by the onset of satiation, and meal frequency is dependent on the duration of satiety (Blundell et al., 1996). Whilst factors relating to nutrient intake and body adiposity determine to some extent the sensations of satiation and satiety, in humans in particular, food intake is not entirely dependent on energy requirements (de Castro, 1997). The interpretation and integration of
all these factors occurs across a network of appetite regulating centres in the brain, a description of which is given in section 1.3.1.

1.2.3 Obesity: Failure of homeostatic regulation of body adiposity

When energy intake exceeds energy expenditure, the result is an accumulation of fat in subcutaneous and visceral adipose depots, and over a prolonged period this can lead to obesity. Both environmental and genetic factors are thought to contribute to this mismatch in energy supply and demand. Rare monogenetic causes of obesity have been identified which lead to severe, early-onset obesity (Yeo et al., 1998; Farooqi et al., 1999; Farooqi et al., 2000; Yeo et al., 2004; Gray et al., 2006; Creemers et al., 2008). Genome-wide association studies (GWAS) have identified other genes, in which mutations are not sufficient to cause obesity in isolation, but are associated with an increase in body weight. One example of such a gene is FTO (fat mass and obesity associated) (Frayling et al., 2007). Homozygous carriers of a particular variant of the FTO gene weigh on average 3kg more than those not carrying the ‘risk’ allele (Frayling et al., 2007). The precise cellular role of FTO is as yet unknown, although may be involved in the regulation of gene expression by DNA demethylation (Gerken et al., 2007). As yet GWAS have failed to identify enough gene variants with sufficient impact on body weight to explain the large contribution genetics is thought to make to obesity (Segal and Allison, 2002; Speliotes et al., 2010).

At the interface of genetic and environmental causes of obesity, epigenetic modifications are thought to contribute to an obese phenotype. Maternal overnutrition has been shown in rodent models to lead to an increased body weight and associated co-morbidities in the offspring, and similar associations have been observed in humans (Rooney and Ozanne, 2011).

An ‘obesogenic’ environment is often cited as the cause for the rise in obesity in recent decades. The environmental changes which are suggested to contribute include reduced physical activity, greater availability of calorie dense/high fat foods, stressful lifestyles, increased time spent watching
television, the presence of endocrine disruptors, reduction in hours spent sleeping, and the prevalence of central heating (Keith et al., 2006). However, despite similar changes in environment across whole populations only a proportion of people become obese suggesting an interaction between genetic and environmental factors is likely to be responsible for the rise in obesity.
1.3 **OVERVIEW OF APPETITE CONTROL SYSTEMS**

The regulation of food intake occurs across a number of interconnected centres in the brain and also requires feedback from peripheral sources providing information about the animal’s energy requirements. The hypothalamus has long been recognised as a crucial appetite regulating centre (Hetherington and Ranson, 1940; Brobeck et al., 1943), and increasingly the importance of centres in the brainstem and the brain’s reward networks is becoming clear (Rinaman, 2010; Berthoud, 2011). Furthermore, the responsibility for monitoring nutrient availability is shared with peripheral centres, such that availability of ingested, circulating and stored nutrients is assessed at the level of the gut, liver and adipose tissue as well as in the brain. Hormonal and nervous signals have been implicated in the regulation of appetite and these signals, together with the central appetite regulating circuits which process them, are discussed below (Figure 1.1) (Gibbs et al., 1973; Woods et al., 1979; Kral, 1981; Halaas et al., 1995; Tang-Christensen et al., 1996; Flint et al., 1998; Tschop et al., 2000; Batterham et al., 2002).

1.3.1 **Neurocircuitry**

The neurocircuitry involved in energy balance must be able to sense regulated parameters, integrate signals from the periphery and within the brain, and effect an appropriate response. The hypothalamus is thought to be at the centre of this system. The hypothalamus sits at the base of the brain and surrounds the third ventricle making it accessible to nutrients and hormones. The ability to sense the internal environment directly via the third ventricle is consistent with the role of the hypothalamus in appetite regulation and homeostasis more generally. Neurons from the brainstem, which receives signals from the peripheral nervous system, project to the hypothalamus, conveying information from peripheral nerve terminals including those in the gut and liver. Alongside these signals the hypothalamus also receives inputs from the cerebral cortex and midbrain reward networks.
The hypothalamus uses the information it receives to determine the energy status of the animal. Hypothalamic output to the pituitary gland, which releases hormones, the brainstem, which controls the autonomic nervous system, and to higher centres in the brain, which control behaviour, form the basis of the control of appetite and energy expenditure.

Figure 1.1: Overview of appetite control systems. Taken from Murphy and Bloom 2006.
1.3.1.1 The Hypothalamus

1.3.1.1.1 Anatomy

The hypothalamus is located in the ventral diencephalon. The ventral portion of the hypothalamus is enclosed within the circle of Willis, which provides its blood supply. The third ventricle divides the hypothalamus along the midline. The median eminence connects the hypothalamus to the pituitary stalk which forms a link between the hypothalamus and the posterior pituitary. Neuroendocrine factors released from the hypothalamus pass via the pituitary stalk to communicate with the pituitary.

The hypothalamus can be divided into three anatomical zones which are somewhat functionally distinct. The lateral zone is the largest and contains the median forebrain bundle, a collection of axon tracts connecting the hypothalamus with both the forebrain and hindbrain. Neurons within the lateral zone are thought to modulate attention and arousal (Swanson, 1987) and the lateral hypothalamic area (LHA) has been implicated in the regulation of food intake (section 1.3.1.1.2.5).

The medial zone receives inputs from limbic centres in the forebrain and amygdala. This zone contains the ventromedial hypothalamic nucleus (VMN), and dorsomedial hypothalamic nucleus (DMN), both of which have roles in regulating feeding, and are thought to be particularly important for coordinating the motivational aspects of the feeding response (Swanson, 2000). The region of the paraventricular nucleus (PVN) thought to be involved in the regulation of feeding also forms part of the medial zone.

The remainder of the PVN and the arcuate nucleus (ARC) form part of the periventricular zone, which is considered the source of the major neuroendocrine output of the hypothalamus. As well as the importance of this zone for feeding, it is crucial to the regulation of the hypothalamic pituitary axis and circadian rhythms (Swanson, 2005).
1.3.1.1.2 Hypothalamic nuclei involved in appetite regulation

1.3.1.1.2.1 The Arcuate Nucleus

The arcuate nucleus (ARC) is located at the base of the hypothalamus abutting the third ventricle. It is thought to be a direct recipient of some systemic hormonal signals as it has receptors for hormones such as leptin (Mercer et al., 1996). Amongst the neuronal populations found in the ARC are neuropeptide Y (NPY) and agouti gene-related protein (AgRP) co-expressing neurons, and pro-opiomelanocortin (POMC) expressing neurons (Hahn et al., 1998; Elias et al., 1998). These two populations are considered first order neurons, and a key part of the melanocortin system: the neuronal network with the best characterised and most widely accepted role in the regulation of energy homeostasis. Post-translational processing of the POMC gene product leads to the
production of α-melanocyte stimulating hormone (α-MSH). α-MSH is an agonist at the melanocortin-4 receptor (MC4R) and activation of this receptor at sites within and beyond the hypothalamus leads to a reduction in food intake (Fan et al., 1997; Zheng et al., 2005c). AgRP also acts at the MC4R, but is both a competitive antagonist and inverse agonist at this receptor, and causes an increase in food intake (Ollmann et al., 1997; Nijenhuis et al., 2001). Thus activation of the AgRP or POMC expressing neurons in the ARC has opposing effects on energy homeostasis. Recently the release of gamma-aminobutyric acid (GABA) by AgRP expressing neurons projecting to the parabrachial nucleus (PBN) has emerged as an important regulator of feeding. Post-natal ablation of AgRP neurons leads to starvation which can be prevented by the administration of a GABA_A receptor agonist into the PBN (Wu et al., 2009; Wu et al., 2012).

1.3.1.1.2.2 The Paraventricular Nucleus

The paraventricular nucleus (PVN) surrounds the anterior dorsal portion of the third ventricle and extends laterally. Populations of magnocellular and parvocellular neurons reside within this nucleus. The magnocellular neurons express oxytocin and vasopressin and project to the pituitary stalk where these peptides are released (Mezey and Kiss, 1991). Within the parvocellular subdivision, are neurons which project to extrahypothalamic regions of the CNS (Swanson and Sawchenko, 1980; Swanson and Kuypers, 1980), and this subdivision is thought to contain neurons which regulate feeding. Some of these neurons release anorexigenic peptides including oxytocin, corticotrophin releasing hormone (CRH) and cholecystokinin (CCK) (Sawchenko and Swanson, 1982; Hokfelt et al., 1990). The PVN is thought to contain second order target neurons of the melanocortin system. The density of projections from NPY/AGRP and POMC neurons of the ARC to the PVN, coupled with high expression of the MC4R, suggest the PVN may be an important downstream target of the melanocortin system (Mountjoy et al., 1994; Cowley et al., 1999; Lu et al., 2003). Discrete lesions of the PVN cause marked hyperphagia and adiposity, as do loss-of-function mutations in the Sim1 gene, a transcription factor necessary for the normal development of the PVN (Leibowitz et al., 1981; Holder, Jr. et al., 2000; Michaud et al., 2001). In addition to playing a role downstream of ARC
activation, the PVN may itself be able to respond to peripheral signals such as leptin (Ghamari-Langroudi et al., 2011).

1.3.1.1.2.3 The Ventromedial Nucleus

The ventromedial nucleus (VMN) is located dorsal to the ARC. The VMN was one of the first nuclei identified with appetite regulation when lesions of this area were found to cause hyperphagia and obesity (Brobeck et al., 1943; Albert et al., 1971). Like the ARC and PVN, the VMN contains leptin receptors (Mercer et al., 1996). Neurons in the VMN may also form part of the melanocortin system. Projections from first order ARC NPY/AgRP and POMC neurons are seen in the VMN, although the projections are not nearly as numerous as those to the PVN (Bagnol et al., 1999). Activation of MC4Rs in the VMN regulates the expression of brain derived neurotrophic factor (BDNF) which is a suppressor of food intake (Kernie et al., 2000; Xu et al., 2003). Projections from the VMN extend to other hypothalamic nuclei (Saper et al., 1976) and the VMN has dense efferent and afferent connections with the amygdala (Krettek and Price, 1978).

1.3.1.1.2.4 The Dorsomedial Nucleus

The dorsomedial nucleus (DMN) is located dorsal to the VMN and, like the ARC, PVN and VMN, is adjacent to the third ventricle. DMN lesioned rats were shown to be hypophagic and lacked the normal feeding response to peripheral glucoprivation (Bernardis, 1970; Bellinger et al., 1978). Glucoprivation has also been shown to induce c-fos (a marker of neuronal activation, see section 3.1.6.2 for details) in the DMN (Briski and Sylvester, 2001). Thus it is possible this region is important for stimulating food intake in response to low glucose levels. The DMN receives inputs from most major hypothalamic nuclei including the PVN, ARC and VMN and from the parabrachial nucleus (PBN) in the brainstem (Thompson and Swanson, 1998). MC4R receptors are expressed in the DMN (Kishi et al., 2003) and administration of α-MSH or AgRP into the DMN inhibits or stimulates food intake respectively (Kim et al., 2000). This suggests the DMN may be a target of first order neurons in the
melanocortin system. The DMN is also a target of circulating leptin, which appears to stimulate thermogenesis in mice by its action at the DMN (Enriori et al., 2011).

The entrainment of circadian rhythms by food availability seems to involve the DMN. Cell specific DMN lesions block the increase in wakefulness, locomotor activity and body temperature seen in anticipation of a meal in schedule-fed rats (Gooley et al., 2006) and restoration of the clock gene Bmal1 in the DMN of mice with a global targeted disruption of this gene is sufficient to restore food entrainable rhythms (Fuller et al., 2008).

1.3.1.1.2.5 The Lateral Hypothalamic Area

The region lateral to the VMH and DMH is known as the lateral hypothalamic area (LHA). Lesions in this area caused hypophagia which lead to its initial designation as a feeding centre (Anand and Brobeck, 1951). Melanin-concentrating hormone (MCH) is expressed by a subset of neurons in the LHA and is orexigenic when injected into the ARC and PVN (Abbott et al., 2003; Naito et al., 1988). MCH neurons in the LHA are thought to form part of the melanocortin system, perhaps acting downstream of NPY/AGRP and POMC neurons which have been shown to contact LHA MCH neurons (Broberger et al., 1998; Elias et al., 1998). MCH administration can block the anorectic effects of α-MSH-stimulated feeding, despite not acting as an antagonist the MC4R, and thus is consistent with MCH action downstream of the MC4R (Ludwig et al., 1998). MCH fibres project widely in the brain including to the entire cerebral cortex and brainstem (Bittencourt et al., 1992).

A separate subset of LHA neurons express the peptides hypocretin 1 and 2 (also known as orexins A and B) (Sakurai et al., 1998). Hypocretin containing neurons project to areas including the ARC, PVN, VMH, PBN, nucleus tractus solitarius (NTS) and area postrema (AP) (Peyron et al., 1998). Central injection of hypocretins is orexigenic in rats (Sakurai et al., 1998). However it is thought that the orexigenic effect of the hypocretins may be secondary to their effects on arousal. These peptides have been found to stimulate activity when injected into the PVN, and chronic central infusions do not lead to an overall increase in food intake or body weight (Haynes et al., 1999; Kiwaki et al., 2004).
1.3.1.2 Brainstem appetite regulating centres

The brainstem is sufficient in isolation from other central appetite regulating centres to effect some feeding and satiety responses. Decerebrate animals retain the ability to cease feeding when satiated, to respond to palatable stimuli and to respond to peripheral glucoprivation by feeding (Grill and Norgren, 1978a; Grill and Norgren, 1978b; Grill and Norgren, 1978c; Flynn and Grill, 1983). However, they are unable to adjust meal size to counter long-term nutrient deprivation (Seeley et al., 1994). Neurons projecting to the brainstem from the hypothalamus may be involved in the brainstem modulation of meal size in response to metabolic status. POMC, oxytocin, hypocretin and MCH expressing neurons have all been shown to project from the hypothalamus to the brainstem (Zheng et al., 2005c; Blevins et al., 2004; Peyron et al., 1998; Buijs et al., 2001). Communication between the hypothalamus and brainstem is bidirectional, with ascending signals from the brainstem to the hypothalamus also involved in regulating feeding (Rinaman et al., 1995; Willing and Berthoud, 1997; Goldstone et al., 2000; Lawrence et al., 2002). However it does appear that the brainstem itself can respond to some circulating signals of long-term metabolic status. Both leptin and ghrelin can signal directly to the brainstem, and leptin can modulate the response of the nucleus tractus solitarius (NTS) to vagal signals (Faulconbridge et al., 2003; Huo et al., 2007).

A brief description of the brainstem centres involved in appetite regulation is given below, with further detail to be found in section 3.1.5.

1.3.1.2.1 The Area Postrema

The AP lies dorsal to the 4th ventricle, has a highly permeable blood brain barrier and as such is classed as a circumventricular organ (Wislocki, 1920). Increased c-fos-like immunoreactivity in the AP is seen in response to circulating anorectic hormones including cholecystokinin (CCK), amylin, peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) (Bonaz et al., 1993; Barth et al., 2004; Fraser and Davison, 1992; Baumgartner et al., 2010). Neurons in the AP project to other areas of the brainstem, including the NTS and PBN (Shapiro and Miselis, 1985b; Lanca and van der Kooy, 1985).
There are also projections from the AP to the PVN and DMN in the hypothalamus (Shapiro and Miselis, 1985b).

1.3.1.2.2 The Nucleus Tractus Solitarius

The NTS receives hormonal and nervous (vagal and non-vagal splanchnic afferent) signals from the periphery pertaining to the short-term energy status of the animal, including the degree of gastric distension and the presence of duodenal nutrients. Extensive projections have been documented from the NTS to the PBN, hypothalamus and amygdala (Ter Horst et al., 1989). Neurons within the hypothalamus also project back to the NTS releasing oxytocin, MCH or α-MSH, and α-MSH injected into the fourth ventricle inhibits food intake (Zheng et al., 2005c; Zheng et al., 2005a).

Modulation of the response of the NTS to signals of short-term nutrient availability, as a consequence of the long-term energy status of the animal, may occur via signalling from the hypothalamus. Projections have been observed from POMC neurons in the ARC to the NTS (Zheng et al., 2005c) and activation of MC4Rs in the NTS has been shown to affect the response to peripherally administered CCK (Sutton et al., 2005). A subset of PVN neurons expressing oxytocin have been shown to project to the NTS and fourth ventricular administration of an oxytocin receptor antagonist is orexigenic (Blevins et al., 2004). MCH neurons in the LHA also project to the NTS, where their axons appear to make close contact with neurons activated by gastric distension and also with tyrosine hydroxylase and GLP-1 expressing neurons in this region, although no effect of MCH administration into the fourth ventricle was seen (Zheng et al., 2005b). Modulation of brainstem responsiveness to short-term satiety signals can also occur by a direct action of leptin on leptin receptors expressed in the NTS (Grill et al., 2002; Huo et al., 2007).

1.3.1.2.3 The Parabrachial Nucleus

The PBN is situated more rostrally than the NTS and is a major target of NTS ascending outputs (Herbert et al., 1990). It also appears to be an important target of ARC AgRP neurons. Inhibitory GABAergic signalling by AgRP neurons in the ARC projecting to the PBN appears crucial to the
initiation of feeding (Wu, Boyle, & Palmiter 2009). It has been suggested that the activity of neurons in the PBN inhibits food intake, and that inputs from the NTS and ARC are integrated at the level of the PBN to regulate feeding (Wu, Clark, & Palmiter 2012).

1.3.1.3 Regulation of appetite by reward centres

Motivation and reward are important in the regulation of most animal behaviour, and feeding is no exception. The expectation of a reward is required to motivate an animal to seek food, and consuming food once obtained must also be rewarding. The neural correlates of reward for feeding appear to be similar to those involved in addictive behaviour, and the two have many factors in common (Kelley and Berridge, 2002).

There is a strong association between the intake or presentation of palatable foods and the activation of reward centres in the brain, and there is even evidence that differences in the response of reward centres to palatable foods can be seen between normal weight and overweight or obese individuals (Park and Carr, 1998; Killgore et al., 2003; Stoeckel et al., 2008; Murdaugh et al., 2012). Two components of reward-driven food intake have been described as ‘wanting’, the incentive to eat, and ‘liking’, the hedonic response to feeding (Berridge, 2004). The neural bases for these two components can be divided roughly into two major systems involved in food mediated reward, the mu-opioid and the mesolimbic dopaminergic systems, which are thought to mediate the ‘liking’ and ‘wanting’ aspects respectively.

1.3.1.3.1 The mu-opioid system

The mu-opioid system has been associated with food ‘liking’ (Berridge, 1996). Administration of specific agonists for the mu-opioid receptor into the nucleus accumbens stimulates food intake (Zhang & Kelley 2000). This effect is particularly associated with the response to palatable foods, which can be mimicked by opioid agonist administration into the nucleus accumbens shell or into the posterior ventral pallidum (Pecina and Berridge, 2000; Smith and Berridge, 2005). Conversely opioid receptor antagonists reduce food intake, and do so most potently in the case of a palatable
food, suggesting that opioid signalling may have a physiological role in stimulating feeding (Holtzman, 1974; Giraudo et al., 1993). The stimulation of feeding by mu-opioid agonists in the nucleus accumbens appears to require activation of the LHA and DMN in the hypothalamus, the basolateral amygdala, the ventral tegmental area in the midbrain, and the NTS, suggesting these are downstream targets of the mu-opioid system (Will et al., 2004; Will et al., 2003).

1.3.1.3.2 The mesolimbic dopaminergic system

The mesolimbic dopaminergic system is thought to stimulate food ‘wanting’, the motivation to acquire food (Berridge, 1996). Dopamine depletion leads to animals being aphagic (Zhou and Palmiter, 1995). Dopaminergic projections originate in the ventral tegmental area (VTA) in the midbrain and project to the nucleus accumbens as well as to other centres including the prefrontal cortex and amygdala which are thought to mediate motivation (Ungerstedt, 1971; Swanson, 1982). Projections from the LHA, and a lesser number from the PVN, VMN and DMN are seen to the VTA (Geisler and Zahm, 2005). Hypothalamic modulation of VTA dopaminergic signalling may occur via LHA leptin receptor expressing neurons (Leinninger et al., 2009), release of hypocretins (Korotkova et al., 2003) or by MC4R signalling in the VTA (Lerma-Cabrera et al., 2012) although no clear mechanism has been established.

The dopaminergic output of the VTA is under some control by circulating factors. Leptin injections into the VTA inhibit food intake (Hommel et al., 2006) while ghrelin injections into the VTA and nucleus accumbens stimulate it (Naleid et al., 2005). PYY3-36 has been shown using fMRI to activate the VTA in humans (Batterham et al., 2007) and the GLP-1 receptor agonist exendin-4 (EX-4) inhibits food intake when injected into the VTA of rats (Alhadeff et al., 2012). Together these findings imply that the effects of circulating hormones on satiety may be partially mediated by a direct effect on the reward system at the level of the VTA.
1.3.2 Gut-Brain signalling

The centres in the brain controlling appetite receive information about nutritional status from the periphery. The presence of food in the gastrointestinal tract is predictive of the future availability of circulating nutrients and thus is an important factor in determining appetite. The body’s requirements for a range of macronutrients necessitate detection of specific macronutrients in the gut and the ability to relay that information to appetite regulating centres.

1.3.2.1 Nutrient sensing

Nutrient sensing can occur either at the level of the gut luminal contents or as a response to substances absorbed across the intestinal epithelium. G-protein coupled receptors (GPCR) on the luminal side of intestinal epithelial or enteroendocrine cells are important for the first of these. One example are the sweet taste receptors, which as well as being present in the oral cavity are also expressed by the intestinal epithelium (Bezencon et al., 2007). A number of GPCRs have been shown to be sensitive to free fatty acids (FFA) and to be expressed on enteroendocrine cells (Liou et al., 2010; Briscoe et al., 2003; Brown et al., 2003; Hirasawa et al., 2005). Peptides and amino acids have also been hypothesised to be detected by GPCRs on the gut luminal wall, although the physiological significance of these receptors is not yet known (Nemoz-Gaillard et al., 1998; Bezencon et al., 2007).

The uptake of nutrients across the intestinal epithelium can be detected as a result of changes in membrane potential induced by the co-transport of charged ions and the absorbed nutrient. For example the sodium glucose co-transporter SGLT1 generates currents by the uptake of glucose in an enteroendocrine cell line (Gribble et al., 2003) and a similar mechanism may exist for peptide and amino acid transporters (Cordier-Bussat et al., 1998).

1.3.2.2 Nervous communication between the gut and brain

Although it is believed there is some direct action of certain gut hormones on the brain, many are thought to act on the brain indirectly via the vagus nerve. Other signals, such as those resulting from activation of taste receptors in the oral cavity or mechanical distension of the gut can also be relayed
by the vagus nerve to the brain. Vagal afferents are present in the mucosa of the stomach which respond to touch, and stretch and tension receptors are present in the external muscle layer throughout the gastrointestinal tract (Berthoud et al., 1997; Phillips and Powley, 2000; Berthoud et al., 2001). Afferents present in the mucosa are thought to respond to locally produced hormones such as CCK, ghrelin and leptin (Wang et al., 1997; Date et al., 2002) and studies using surgical or chemical subdiaphragmatic vagotomy have suggested that the vagus nerve is the major route of communication between many gut hormones and the brain (Smith et al., 1985; Date et al., 2002; Abbott et al., 2005). Outside of the gastrointestinal tract, vagal afferents innervating the liver and portal vein have been implicated in nutrient sensing and their signals appear to influence food intake (Friedman, 1997; Horn et al., 2001).

Despite the evidence suggesting the vagus nerve is important for meal termination in response to ingested food, a permanent loss of this feedback mechanism by vagotomy or deafferentation does not appear to lead to increased body weight, an effect that is thought to be due to compensation by reduced meal frequency (Schwartz et al., 1999; Powley et al., 2005).

1.3.2.3 Gut Hormones

Gut hormones are released by endocrine cells within the gut mucosa in relation to food intake. Most of these hormones are released in response to food intake and act in the short term to encourage meal termination. Ghrelin levels are inversely correlated with body mass index and as such are thought to act as a long-term signal of energy status (Shiiya et al., 2002). The actions of the major gut hormones involved in appetite regulation are summarised in Table 1.1.

This thesis focuses on the action of the gut hormones GLP-1 and oxyntomodulin as well as the related pancreatic hormone glucagon. A detailed description of the structure, release and actions of these peptides is given in section 1.4.
<table>
<thead>
<tr>
<th>Name</th>
<th>Origin</th>
<th>Receptors</th>
<th>Location of action</th>
<th>Effect on energy balance</th>
<th>Other roles</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholecystokinin</td>
<td>I cells in proximal small intestine</td>
<td>CCK1, CCK2</td>
<td>vagus nerve brainstem hypothalamus</td>
<td>Reduces food intake</td>
<td>Neuropeptide transmitter in the CNS</td>
<td>(Sjolund et al., 1983; Baldwin et al., 1998)</td>
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<td>(CCK)</td>
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<tr>
<td>Peptide YY</td>
<td>L cells in distal small intestine and colon</td>
<td>Y2 (PYY3-36) Y1, Y2, Y5 (PYY1-36)</td>
<td>ARC Vagus nerve Brainstem</td>
<td>Reduces food intake</td>
<td></td>
<td>(Adrian et al., 1985; Batterham et al., 2002)</td>
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<tr>
<td>(PYY)</td>
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<tr>
<td>Pancreatic Polypeptide</td>
<td>PP cells in the pancreatic islets</td>
<td>Y4 Y1 Y5</td>
<td>Brainstem ARC PVN</td>
<td>Reduces food intake increases energy expenditure</td>
<td></td>
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<td>(PP)</td>
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<tr>
<td>GLP-1</td>
<td>L cells in distal small intestine and colon</td>
<td>GLP-1R</td>
<td>Brainstem PVN VMN Vagus?</td>
<td>Reduces food intake</td>
<td>Neuropeptide transmitter in the CNS Peripheral insulin release</td>
<td>(Orskov et al., 1988) (Tang-Christensen et al., 1996; Larsson et al., 1975) (Adrian et al., 1976) (Asakawa et al., 1999) (Liu et al., 2008)</td>
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<tr>
<td>Oxyntomodulin</td>
<td>L cells in distal small intestine and colon</td>
<td>GLP-1R GCGR</td>
<td>ARC PVN Supraoptic nucleus</td>
<td>Reduces food intake increases energy expenditure</td>
<td></td>
<td>(Baggio et al., 2004b; Dakin et al., 2001)</td>
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<tr>
<td>(OXM)</td>
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<tr>
<td>Ghrelin</td>
<td>A-like cells in the gastric oxyntic glands</td>
<td>GHS-R1a ARC Vagus VTA</td>
<td>Increases food intake</td>
<td>Growth hormone secretagouge</td>
<td></td>
<td>(Asakawa et al., 2001)</td>
</tr>
</tbody>
</table>

**Table 1.1: Summary of characteristics of gut hormones affecting appetite.** CCK, cholecystokinin; PYY, peptide YY; PP, pancreatic polypeptide; GLP-1, glucagon like peptide 1; GLP-1R, glucagon like peptide 1 receptor; GCGR, glucagon receptor; GHS-R1a, growth hormone secretagouge receptor 1a; OXM, oxyntomodulin; ARC, arcuate nucleus; PVN, paraventricular nucleus; VMN, ventromedial nucleus.
1.4 **The Preproglucagon Derived Peptides**

1.4.1 The Preproglucagon Gene

The preproglucagon (PPG) gene encodes for the peptide hormones glucagon, GLP-1, GLP-2, oxyntomodulin and glicentin (Lund et al., 1982). The gene is transcribed producing the PPG protein, which is cleaved to proglucagon by the removal of an N terminal signal sequence. In mammals PPG is transcribed as a single mRNA molecule and the different peptides are produced by post-translational processing. Tissue specific peptide production is determined by the expression of enzymes which cleave the proglucagon peptide in different ways (Figure 1.3). In other vertebrates, alternative splicing of the PPG gene occurs and multiple mRNA molecules can be produced in different tissues, thus the regulation of peptide expression is very different to that in mammals.

![Proglucagon Processing Diagram](image_url)

**Figure 1.3:** Proglucagon processing in the pancreas and intestinal tract. Taken from Holst 2007 (Holst, 2007).

The PPG gene in mammals is a single copy gene present on chromosome 2 in humans, but in some species, such as the anglerfish, duplication of the gene has occurred and they carry two copies. The
sequence for glucagon is thought to have been duplicated in two independent events to produce the sequences for GLP-1 and GLP-2. The first duplication is thought to have occurred 1150 million years ago producing what is now the sequence for GLP-2 (Lopez et al., 1984). The second duplication, again of the glucagon sequence, produced the sequence for GLP-1 around 800 million years ago. The diversification of vertebrates, occurring 500 million years ago meant that the evolution of PPG from this point onwards in mammals and other vertebrates occurred independently. The GLP-2 sequence was lost from the ancestral anglerfish and thus GLP-2 is not present in several species. The duplication of the PPG gene in the anglerfish occurred after the loss of the GLP-2 sequence about 160 million years ago and thus two PPG sequences are present in amphibians, bony fish and jawless fish today (Irwin, 2001).

The sequence of glucagon is invariant at eight amino acid positions (1, 4, 6, 9, 10, 12, 25, and 26) across all vertebrates (Irwin, 2001) which do not correlate with those positions thought most crucial for receptor binding, suggesting that receptor binding may occur differently across the species. The length of glucagon is largely invariant at 29 amino acids. The length of GLP-1 varies much more between species ranging from 28 to 38 amino acids and also contains fewer invariant amino acids than glucagon, with only 5 (1, 2, 4, 7, and 22). Two of the invariant amino acids represent positions crucial to receptor activation in mammals. The greater variability in GLP-1 sequence is thought to reflect its more diverse roles in different species.

In mammals GLP-1 is secreted from L-cells in the intestine and is a mediator of the incretin effect, the increased insulin release seen in response to orally administered glucose as compared to intravenous glucose (Schmidt et al., 1985; Orskov et al., 1986). In fish, both intestinal and pancreatic cells secrete GLP-1, which has similar hyperglycemic effects to glucagon and binds to a receptor more closely related to the glucagon receptor than to the GLP-1 receptor in mammals (Plisetskaya and Mommsen, 1996; Irwin and Wong, 2005). Thus GLP-1 and glucagon function in humans must be
studied in a mammalian model and the remainder of this thesis will focus on the function and utility of the PPG derived peptides in rodent models.

1.4.2 Glucagon

1.4.2.1 Peptide

Glucagon is a 29 amino acid peptide produced in the pancreatic α-cells and is a product of the PPG gene. Proglucagon is cleaved by prohormone convertase 2 (PC2) to produce glucagon (Rouille et al., 1994) (Figure 1.3). Outside the pancreas low levels of glucagon expression are detectable using immunohistochemistry in the hypothalamus and brainstem (Larsen et al., 1997a) although it has no known role in the CNS.

1.4.2.2 Stimuli for Glucagon secretion

Glucagon is released under fasting conditions. Low glucose concentrations lead to the closure of K\textsuperscript{+}ATP channels in pancreatic islet α-cell membranes (Olsen et al., 2005). This prevents the efflux of K\textsuperscript{+}, and the resulting membrane depolarisation triggers release of granules containing glucagon (Gromada et al., 1997a). Glucagon release from isolated islet preparations is pulsatile, and glucose concentration affects the amplitude, but not the frequency, of glucagon secretion (Opara et al., 1988).

Glucagon release can also be stimulated by central glucopenia. Local hypoglycaemia, induced by infusion of 2-deoxyglucose into the VMN, increases blood glucagon levels (Borg et al., 1995). Conversely, direct delivery of glucose into the VMN significantly reduces glucagon release (Borg et al., 1997). The hypothalamus can stimulate the release of glucagon from pancreatic α-cells via sympathetic nerves (Wu et al., 2004a). There may also be a role for the hindbrain in regulating glucagon secretion; local hypoglycaemia in the hindbrain leads to a rise in circulating glucagon concentration (Andrew et al., 2007).

Glucagon release is not restricted to conditions of low blood glucose. Glucagon is released at meal onset, an effect which is thought to be mediated by noradrenergic signalling from the VMN (De Jong
A. et al., 1977). Activity of noradrenergic projections from the hypothalamus, and the resultant activation of α1- and β-adrenergic receptors on pancreatic α-cells, is also thought to be responsible for the release of glucagon in response to stress (Bloom and Edwards, 1975).

1.4.2.3 The Glucagon Receptor
The glucagon receptor (GCGR) is a member of G protein-coupled receptor (GPCR) family B (Jelinek et al., 1993). Ligand binding leads both to adenylate cyclase and phospholipase C activation (Wakelam et al., 1986). GCGR mRNA is expressed at highest levels in the liver, heart, adipose tissue and kidney but is also expressed throughout the GI tract and in the spleen, thymus and adrenals at relatively high levels. Lower levels of expression are seen in the pancreatic islets, lung and cerebral cortex (Hansen et al., 1995; Dunphy et al., 1998). More specific analysis of GCGR expression within the CNS has not been carried out although ^125_I –labelled glucagon has been shown to bind specifically, in competitive binding assays with unlabelled glucagon, to isolated membranes from central sites including the hypothalamus, amygdala, hippocampus and anterior pituitary (Hoosein and Gurd, 1984).

1.4.2.4 Physiological Roles of Glucagon

1.4.2.4.1 Glucose Homeostasis
Glucagon is best known as the counterregulatory hormone to insulin: raising blood glucose in times of fasting. To bring about a rise in blood glucose, glucagon increases the rate of gluconeogenesis and the breakdown of glycogen stores. For a description see section 4.1.2.3.2.1.

1.4.2.4.2 Regulation of Food Intake
Peripherally administered glucagon induces postprandial satiety (Geary, 1990). The mechanisms for this are unknown. Potential mechanisms are discussed in section 3.1.3.

The extent of the physiological role of endogenous glucagon in reducing food intake is unclear. The dose of peripherally administered glucagon required to inhibit food intake appears to be supraphysiological. Intravenous (IV) doses between 5 and 50 μg/kg of glucagon are required
(calculated from figures collated in (Geary, 1996). Assuming a 250g rat with a plasma volume of 10.4 ml (Lee and Blaufox, 1985) the plasma level after this dose is administered would be between 0.12 μg/ml and 1.2 μg/ml. By contrast the physiological plasma concentration in rats varies from 50-125 pg/ml (Ruiter et al., 2003).

It has been suggested that the observed discrepancy between physiological glucagon concentrations, and those required to reduce food intake, might be explained by the pulsatile release seen physiologically. However, administering glucagon in two separate one-minute pulses (designed to mimic the natural pulsatile rhythm of secretion) does not enhance the satiating effect of a 14 μg dose when compared to continuous infusion (Geary, 1996). It may be that the local concentration of glucagon at its site of action is higher than that seen in the peripheral circulation. A direct comparison of the levels of glucagon in the portal circulation post-prandially and after exogenous administration of a satiating dose is required to determine whether the effect can be considered physiological.

Notwithstanding these observations, infusions of glucagon antibodies into the portal circulation of rats during meals cause increased food intake, implying endogenous glucagon suppresses food intake during meals (Le Sauter J. et al., 1991). A rise in circulating glucagon is seen post-prandially in rats under scheduled feeding conditions, where the rise lasted 0.5 to 1.5 hours after each meal (Ruiter et al., 2003).

1.4.2.4.3 Energy Expenditure

Glucagon increases energy expenditure. Rats treated with glucagon lose more weight than pair fed saline controls (Salter, 1960) and glucagon dose-dependently increases oxygen consumption (Davidson et al., 1960). The mechanism for this increase is not fully understood. Subcutaneous administration of 1 mg/kg glucagon twice daily for 18 days increases oxygen consumption and growth of brown adipose tissue (BAT) (Billington et al., 1987; Billington et al., 1991). Injections of glucagon into the LHA increase sympathetic activity to the interscapular BAT in rats (Shimizu et al.,
1993) which might suggest glucagon acts directly on the hypothalamus to increase sympathetic nerve activity. Glucagon also stimulates lipolysis in white adipose tissue (Richter et al., 1989).

It is difficult to determine whether glucagon plays a physiological role in weight regulation. The obese Zucker rat has a mutation in the leptin receptor and these rats have reduced blood glucagon concentration (Eaton et al., 1976). Administration of glucagon (0.33 mg/kg) twice daily to obese Zucker rats reduces body weight gain by 20% over a 9 month period compared to untreated controls (Chan et al., 1984). This suggests that the reduced glucagon levels in this model contribute to the obese phenotype. However, the relevance of this observation in a genetically abnormal animal to normal physiology may be limited. GCGR knockout mice, contrary to glucagon’s proposed action to inhibit appetite and increase energy expenditure, are lean and have reduced WAT weight (Gelling et al., 2003). However, GCGR knockouts do exhibit impaired responses to fasting in that they do not increase their fatty acid utilization (Longuet et al., 2008). This would imply that glucagon’s ability to increase energy expenditure may be more important as an acute response to fasting rather than in chronic body weight regulation.

1.4.2.4.4 Glucagon, Stress and the Hypothalamic-Pituitary-Adrenal Axis

Glucagon is released in response to a variety of physical and psychological stressors (Bloom et al., 1973) and its release can be considered as an important part of the stress response (Jones et al., 2012). Aside from increasing substrate availability, glucagon also affects the rate and force of cardiac contractility (Regan et al., 1964). Both of these effects are conducive with facilitation of a fight or flight response.

Glucagon in turn affects the activity of the hypothalamic-pituitary-adrenal (HPA) axis. Activation of the HPA axis is one mechanism by which glucagon might influence appetite (Figure 1.4). However the effects of glucagon on the HPA axis seem to be species dependent. Glucagon causes adrenocorticotropic hormone (ACTH) and cortisol elevation in humans, but suppresses ACTH release in rats (Waldhausl et al., 1976; Rao, 1995). There are no published data for mice. However, despite
having opposite effects on the HPA axis, glucagon inhibits food intake in both rats and humans (Schulman et al., 1957; Salter, 1960), and thus, it could be argued that the modulation of HPA axis activity by glucagon is unlikely to contribute to its anorectic effect. A further possibility is that glucagon inhibits food intake by interaction with the HPA axis in the hypothalamus. Corticotropin releasing hormone (CRH) is released from hypothalamic neurons and central injection of CRH inhibits feeding in rats (Benoit et al., 2000). There is evidence in chicks that hypothalamic CRH mRNA expression is upregulated by glucagon, but no equivalent study has been carried out in mammals (Honda et al., 2007).

**Figure 1.4: Summary of interaction between glucagon and the HPA axis.** Solid arrows indicate stimulation, dashed arrows indicate inhibition. CRH-corticotropin releasing hormone, ACTH-adrenocorticotropic hormone.
1.4.3 GLP-1

1.4.3.1 Peptide

GLP-1 is also formed by tissue specific post-translational processing of the peptide proglucagon (Mojsov et al., 1986). There are two active circulating forms of GLP-1, GLP-17-37 and GLP-17-36-NH2, which have identical effects in vivo (Orskov et al., 1993). In intestinal L-cells, from which GLP-1 is released, proglucagon is cleaved by prohormone convertase 1/3 (PC1/3) to produce GLP-1 (Mojsov et al., 1987; Rouille et al., 1997). L-cells are mainly found in the distal jejunum, ileum and colon (Eissele et al., 1992). Preproglucagon is also expressed in the CNS and GLP-1 immunoreactivity is found in neuronal cell bodies in the caudal NTS and nerve terminals in the hypothalamus, notably in the PVN and DMH (Drucker and Asa, 1988; Larsen et al., 1997a).

1.4.3.2 Stimulation of GLP-1 secretion

GLP-1 is secreted by L-cells at a low level during fasting conditions (Toft-Nielsen et al., 1996). More substantial GLP-1 release occurs post-prandially and is thought to be stimulated directly by nutrients in the intestine (Herrmann et al., 1995). Phospholipids and FFAs in the intestine bind with varying affinities to GPR40, GPR119 and GPR120, leading to GLP-1 release (Hirasawa et al., 2005; Edfalk et al., 2008; Chu et al., 2008). L-cells are glucose sensitive, and depolarise in response to glucose entry, causing GLP-1 release, although the exact mechanism is debated (Parker et al., 2010). Proteins and amino acids are weaker stimuli for GLP-1 release than carbohydrates and fats (Elliott et al., 1993). Peptide uptake by proton coupled cotransporters is thought to trigger depolarisation of L-cells stimulating GLP-1 secretion (Matsumura et al., 2005). In addition, anticipatory release of GLP-1 before nutrients reach the intestine has been observed in meal fed rats (Vahl et al., 2010). Neural control may be responsible for anticipatory GLP-1 release, as muscarinic agonists and noradrenaline stimulate and inhibit GLP-1 release respectively from isolated porcine ileum (Hansen et al., 2004).
1.4.3.3 The GLP-1 Receptor

The GLP-1 receptor (GLP-1R), like the glucagon receptor is a member of GPCR family B. Ligand binding leads to activation of adenylate cyclase (Thorens, 1992). GLP-1R expression is highest in the lung and is also found in the pancreatic islets, kidney, heart, stomach and brain (Bullock et al., 1996). CNS expression of the GLP-1R includes numerous centres involved in energy balance, notably the PVN, ARC and DMN in the hypothalamus and the NTS, AP and PBN in the brainstem (Merchenthaler et al., 1999). The GLP-1R is also present on hepatic vagal neurons (Nakagawa et al., 2004).

1.4.3.4 Physiological roles of GLP-1

1.4.3.4.1 Glucose homeostasis

GLP-1 is an incretin, acting at pancreatic β-cells to increase glucose-stimulated insulin release (Mojsov et al., 1987). GLP-1 binding to nodose ganglion neurons may also be important for insulin release as ganglionic blockers reduce the incretin effect of portal GLP-1 (Balkan and Li, 2000). GLP-1 also inhibits glucagon release (Orskov et al., 1988) and gastric emptying (Young et al., 1996). Thus GLP-1 exerts its effect on blood glucose by reducing the rate at which glucose enters the bloodstream following a meal, as well as by increasing insulin secretion (Nauck et al., 1997). Glucose homeostasis is impaired in GLP-1R knockout mice (Scrocchi et al., 1996) (see section 3.1.6.3.2).

1.4.3.4.2 Regulation of Food Intake

GLP-1 induces satiety when administered peripherally (Flint et al., 1998) or centrally (Tang-Christensen et al., 1996), yet evidence for the physiological role of endogenous GLP-1 in appetite regulation is inconsistent. Administration of the GLP-1R antagonist exendin 9-39 (EX 9-39) causes hyperphagia in rats at times of day when food intake is normally low. In addition, EX 9-39 prevents the reduction in sucrose intake by rats after a sucrose preload, indicating that EX 9-39 reduces the extent of satiety in response to sucrose (Williams et al., 2009). However GLP-1R knockout mice display normal food intake and body weight (Scrocchi et al., 1996) and EX 9-39 has been shown to be
insufficient to reduce satiety in response to a meal in rats (Ruttimann et al., 2010) thus the physiological role of GLP-1 in regulating appetite is yet to be confirmed.

The mechanisms of GLP-1 induced anorexia are discussed in section 3.1.2. The effects of peripherally administered GLP-1 appear to be mediated by GLP-1Rs expressed in the periphery as GLP-1-induced c-fos expression in the ARC is abolished by vagotomy (Abbott et al., 2005) and albumin-conjugated GLP-1, which cannot cross the blood brain barrier, induces the same pattern of c-fos activation as native GLP-1 (Baggio et al., 2004a). A peripheral site of action for L-cell derived GLP-1 is consistent with the rapid degradation of active GLP-1, in both the gut itself, and in the liver which is thought likely to prevent L-cell derived GLP-1 from reaching the brain (Hansen et al., 1999; Deacon et al., 1996).

1.4.3.4.3 Neurotransmitter

Although peripherally administered GLP-1 does not appear to act directly on the CNS to affect appetite, GLP-1 does act as a neurotransmitter within appetite regulating circuits. Within the NTS, GLP-1 is produced in a population of non-catecholaminergic neurons (Larsen et al., 1997a). These neurons project heavily to the PVN and DMN, with lesser numbers projecting to the ARC, LH and central nucleus of the amygdala (CeA) (Larsen and Holst, 2005). Tonic release of GLP-1 within the CNS may cause suppression of appetite since repeated ICV injection of EX 9-39 increases food intake (Meeran et al., 1999). Administration of EX 9-39 into the 4th ventricle blocks satiety induced by gastric distension, implicating GLP-1 signalling within the brainstem as a mediator of neurally-induced satiety (Hayes et al., 2009).

1.4.3.4.4 GLP-1 and Stress

GLP-1 produced by neurons in the CNS may have a role in the stress response. ICV administration of GLP-1 to rats induces c-fos expression in CRH expressing neurons in the PVN and increases circulating corticosterone and vasopressin concentrations (Larsen et al., 1997b). GLP-1 administration directly into the PVN was found to increase circulating ACTH and corticosterone
concentrations (Kinzig et al., 2003). Central administration of a GLP-1R antagonist blocked the release of stress hormones by LiCl or exposure to vertical heights, suggesting central GLP-1 is involved in the stress response (Kinzig et al., 2003). Consistent with this, GLP-1 expressing neurons were found to be activated by LiCl injection, and some of these neurons project to the PVN (Rinaman, 1999b). GLP-1R knockout mice were found to have altered stress responses, although unexpectedly the corticosterone release in response to environmental stressors was elevated in GLP-1R knockout mice (MacLusky et al., 2000). This effect is consistent with findings that GLP-1 inhibited ACTH-stimulated corticosterone release from dispersed rat adrenocortical cells (Andreis et al., 1999), and may suggest circulating and centrally produced GLP-1 might have differing effects on the stress axis.

1.4.4 Oxyntomodulin

1.4.4.1 Peptide

Oxyntomodulin is a 37 amino acid peptide which like GLP-1 and glucagon, is a post translational cleavage product of PPG. Oxyntomodulin contains the entire sequence of glucagon with an additional eight amino acids at the carboxy-terminus (Bataille et al., 1981b; Holst, 1982). It is produced by cleavage of PPG by prohormone convertase 1/3 and thus is expressed in the same cells as GLP-1. Oxyntomodulin is released concurrently with GLP-1 and the other PPG derived peptides from L-cells in response to nutrients in the intestine and to nervous signals.

1.4.4.2 Receptors for Oxyntomodulin

Oxyntomodulin has no known receptor of its own, but binds to both the GLP-1 and glucagon receptors, albeit with lower affinity than these peptides themselves (Bataille et al., 1981a; Baldissera et al., 1988; Schepp et al., 1996; Dakin et al., 2001; Druce et al., 2009). Oxyntomodulin has around 100 fold lower affinity for the GLP-1R than GLP-1, and around 50 fold lower affinity for the glucagon receptor than glucagon. The GLP-1R is thought to be responsible for the anorectic effect of oxyntomodulin as this is abolished in GLP-1R knockout mice but not in glucagon receptor knockout mice (Baggio et al., 2004b). However some actions of oxyntomodulin may occur independently of
the GLP-1R. For example oxyntomodulin increases intrinsic heart rate in both wild type and GLP-1R knockout mice (Sowden et al., 2007). There is also some evidence that the effects of GLP-1 and oxyntomodulin are divergent (Anini et al., 2000; Baggio et al., 2004b; Chaudhri et al., 2006; Parkinson et al., 2009; Koole et al., 2010). Activation of other receptors, perhaps the glucagon receptor, or an as yet unidentified oxyntomodulin selective receptor, may be responsible for the different actions of these two hormones.

1.4.4.3 Physiological Roles of Oxyntomodulin

The physiological significance of oxyntomodulin in any context has been difficult to assess experimentally. As an agonist at the GLP-1 and glucagon receptors, and with no known receptor of its own, receptor knockout models do not allow us to specifically study the effects of a loss of oxyntomodulin signalling. Furthermore, as this peptide is a product of the PPG gene, containing the entire sequence of glucagon, specific genetic oxyntomodulin knockout models cannot be produced. Despite these caveats numerous studies have been carried out to assess the effects of oxyntomodulin in rodents and in humans, and like its family members glucagon and GLP-1, the effects of oxyntomodulin are seen in the regulation of appetite, energy expenditure and glucose homeostasis.

1.4.4.3.1 Glucose homeostasis

As would be predicted from its activation of both the GLP-1 and glucagon receptors, oxyntomodulin causes insulin release (Jarrousse et al., 1984). Administration of oxyntomodulin to diet induced obese (DIO) mice caused an improvement in their glucose tolerance (Parlevliet et al., 2008). It appears likely that the GLP-1R mediated effects of oxyntomodulin dominate such that there is no hyperglycemic effect (Du et al., 2012). See section 4.1.2.4 for further discussion.

1.4.4.3.2 The Regulation of Food Intake

Oxyntomodulin is anorectic when administered either peripherally or centrally in rodents, as well as peripherally in humans (Dakin et al., 2001; Cohen et al., 2003; Dakin et al., 2004; Wynne et al., 2005).
Oxyntomodulin levels in the blood rise post-prandially (Le Quellec A. et al., 1992) consistent with an action as a satiety hormone. However as the GLP-1R knockout mouse has normal body weight and food intake (Scrocchi et al., 1996) it might be suggested that neither oxyntomodulin nor GLP-1 is important for physiological satiety. Pharmacological blockade of the GLP-1R, used as an alternative to a germline GLP-1R knockout mouse to bypass the problem of developmental compensation, has shown inconsistent effects on spontaneous food intake, with one study finding EX 9-39, increased spontaneous meal size, and another finding it did not (Williams et al., 2009; Ruttimann et al., 2010). Aside from the inconsistency in the findings from these studies it is also impossible to separate any effects of GLP-1 and oxyntomodulin when using GLP-1R blockade. Administration of a specific antibody to oxyntomodulin might be one way to tackle this question but it has not yet been attempted. The mechanisms of oxyntomodulin induced anorexia are discussed in section 3.1.4.

1.4.4.3.3 Energy expenditure

Like glucagon, oxyntomodulin has been shown to increase energy expenditure in both rodents and humans (Dakin et al., 2002; Wynne et al., 2006). GLP-1R activation appears to reduce energy expenditure (Hwa et al., 1998; Baggio et al., 2004b) and thus it is likely oxyntomodulin acts via the glucagon receptor to increase energy expenditure. However there are currently no published studies examining the effects of oxyntomodulin on energy expenditure in glucagon receptor knockout mice, which would be useful in determining whether glucagon receptor activation by oxyntomodulin is indeed the mechanism by which it increases energy expenditure.
1.5 Overview of Thesis

The experiments contained within this thesis aimed to investigate the effects of glucagon, GLP-1 and oxyntomodulin on appetite and glucose homeostasis and their mechanisms. Agonists of both the GLP-1 and glucagon receptors are being investigated as potential treatments for type 2 diabetes and obesity and GLP-1R agonists are already on the market as a treatment for type 2 diabetes. This thesis aimed both to further our understanding of the mechanisms behind the clinically important effects of these peptides and to test new agents based on these hormones being developed in this laboratory.

The first chapter of this thesis examines the effects of glucagon and GLP-1 on feeding in rodents, both individually and in combination. In addition, the acute and chronic effects on food intake and body weight of synthetic agonists designed in this laboratory were studied. Finally the effects of chronic co-administration of these synthetic agonists on body weight and glucose tolerance were investigated in a diet-induced obese mouse model.

The second chapter focuses on the mechanisms by which glucagon, GLP-1 and oxyntomodulin inhibit feeding. The activation of central appetite regulating centres by the three peptides was examined and compared, with a particular focus on the activation of neuronal populations in the nucleus tractus solitarius (NTS). Models of pharmacological and genetic blockade of the GLP-1R were used to assess the importance of this receptor in mediating the appetite inhibiting effects of the preproglucagon derived peptides.

The third chapter in this thesis investigates the effects of glucagon and oxyntomodulin on glucose homeostasis in mice, and the mechanism by which these hormones act to affect blood glucose and insulin. Once again pharmacological and genetic models were used to assess the importance of the GLP-1R in mediating these effects.
2 EXPERIMENTAL CHAPTER ONE

The effects of glucagon and GLP-1 on food intake and the potential of GLP-1/glucagon receptor co-agonists as treatments for obesity
2.1 Pharmacotherapies for Obesity

Epidemiological data on obesity and type 2 diabetes attest the development of efficacious pharmacotherapies are an urgent and profitable target for pharmaceutical companies: 500 million obese adults globally in 2008, predicted to rise to 700 million by 2015 and over 300 million adults with type 2 diabetes worldwide in 2011 (World Health Organisation, 2011a; World Health Organisation, 2011b). Although lifestyle interventions can be effective in causing moderate weight loss of between 5 and 10%, evidence suggests that in all but a small proportion of people, the lost weight is regained within 5 years (NIH Technology Assessment Conference Panel, 1993). While several treatments have been developed to treat type 2 diabetes, there is only one dedicated agent, orlistat, an intestinal lipase inhibitor, currently marketed to treat obesity.

One explanation for this lack of anti-obesity agents lies with the stringent approval criteria set out by the US Food and Drug Administration (FDA) who demand that to be approved as a treatment for obesity an agent must cause a minimum of 5% weight loss above that of a placebo control after 1 year of treatment (Food and Drug Administration, 2007). The European Medicines Agency (EMA) have similar guidelines and also stipulate that weight should not be regained after the therapy is withdrawn (European Medicines Agency., 2007). Both the FDA and EMA also require evidence of reduction in obesity-associated co-morbidities to show that an agent is of clinical, rather than purely cosmetic, value. In addition to the demands placed on efficacy of weight loss agents by regulatory bodies, it is also likely that minimal side effects will be tolerated due to the likelihood that any anti-obesity agent would have to be taken over a long period of time and perhaps for the remainder of the patient’s life to prevent weight regain.

The approaches to producing an anti-obesity agent fall into three broad categories. Firstly there are agents that reduce appetite and therefore energy intake. Secondly there are agents that reduce the efficiency with which consumed nutrients are absorbed, effectively also reducing energy intake. Thirdly there are agents that increase energy expenditure.
Orlistat (Alli, Roche) falls into the second category. It is an analogue of the lipase inhibitor lipstatin, which is produced by *Streptomyces toxytricini*. Orlistat acts locally to inhibit pancreatic and gastric lipase, reducing dietary triglyceride hydrolysis and thus the absorption of dietary fats by around a third. In long-term randomised clinical trials orlistat in combination with diet and exercise has been shown to induce weight loss of 2-4kg more than diet and exercise alone (Rossner et al., 2000; Heymsfield et al., 2000; Torgerson et al., 2004). However diarrhoea, bloating, abdominal pain and dyspepsia are common side effects and limit patient compliance.

Several agents have been, and continue to be, developed in the appetite suppressant category. Despite this none are currently marketed, although two have recently received FDA approval. Appetite suppressants are largely developed either as analogues of anorectic gut hormones or as agents acting directly in the CNS to affect appetite regulating centres in the brain.

The serotonin system has been extensively studied in relation to appetite regulation (Breisch et al., 1976; Tecott et al., 1995; Bouwknecht et al., 2001; Woolley et al., 2001; Heisler et al., 2006; Pringle et al., 2008; Xu et al., 2008; Xu et al., 2010). Sibutramine (Reductil, Abbott) is a serotonin-noradrenaline reuptake inhibitor which was licensed by the FDA in 1997 for the treatment of obesity but was subsequently withdrawn from the market in 2010 following the Sibutramine Cardiovascular Outcomes Trial (SCOUT) which showed patients on sibutramine to have a higher risk of cardiovascular disease than placebo-treated controls (James et al., 2010).

Fenfluramine/phentermine (Redux, Wyeth) combined fenfluramine, a serotonin reuptake inhibitor with phentermine, an amphetamine-like agent. The combination was never approved by the FDA for the treatment of obesity, but was commonly prescribed ‘off-label’ until reports of damage to heart valves associated with fenfluramine lead to its withdrawal by the FDA (Food and Drug Administration, 1997).

In addition to reuptake inhibitors, serotonin receptor agonists have also been developed with the aim of treating obesity. Selective agonists for particular subclasses of serotonin receptors may be
associated with lesser side effect profiles than sibutramine. Locaserin (BELVIQ, Arena Pharmaceuticals), a selective 5-HT₂C receptor agonist, gained FDA approval in June 2012 (Food and Drug Administration, 2012b). Locaserin was previously rejected by the FDA due to concerns about safety (Arena Pharmaceuticals, 2010) and Arena must conduct a long-term cardiovascular outcomes trial once the drug has been marketed. Due to the involvement of serotonin in diverse central and peripheral systems it seems likely that even with receptor selective agents, the side effect profile of agents targeting the serotonin system will present a challenge for an anti-obesity agent.

Rimonabant (Acomplia, Sanofi-Aventis) is a selective CB1 receptor antagonist which, despite being rejected by the FDA, was approved by the EMA for the treatment of obesity until 2009. Central endocannabinoids are thought to stimulate orexigenic pathways in the hypothalamus (Verte et al., 2005; Jo et al., 2005), modulate the rewarding value of food in the nucleus accumbens and affect the response of the brainstem to peripheral satiety signals (Tsou et al., 1998). However rimonabant is associated with depressed mood disorders and anxiety (Christensen et al., 2007). These adverse effects were also seen with another CB1 receptor antagonist taranabant (MK-0364, Merck) (Kipnes et al., 2010). This has lead to the belief that central CB1 receptor antagonists are necessarily associated with psychiatric adverse effects and the cannabinoid system is unlikely to represent a good target for anti-obesity therapies.

Another centrally acting agent, yet to come to market, but which gained FDA approval in July 2012 is Qsymia (formerly known as Qnexa, Vivus Inc.) (Food and Drug Administration, 2012a). Qsymia is a combination of two agents, phentermine, an amphetamine-like compound, and topiramate, an anticonvulsant. Amphetamines have long been known to suppress appetite and are thought to act by increasing levels of dopamine and norepinephrine in the brain (Chen et al., 2001; Leibowitz, 1975). The mechanisms of appetite suppression by topiramate are poorly understood. Qsymia produced significant weight loss in phase III trials, with 70% of patients on the higher dose achieving ≥5% and 48% achieving ≥ 10% weight loss in a one year period (Gadde et al., 2011). However an increase in
depressive symptoms has been reported in patients taking Qsymia and cardiovascular side effects are also a concern (Cohen, 2011).

Agonists and antagonists of central neurotransmitters are likely to elicit unwanted effects due to the diverse functions of these transmitters in the brain. Pharmacological manipulation of these systems alters not only the levels of neurotransmitter or receptor stimulation at synapses in appetite regulating circuits, but those throughout the brain. For this reason peptide hormones may present a better target. Peripherally released peptide hormones affecting appetite are unsuitable as therapeutics due to their short circulating half lives. However, if long-acting synthetic analogues can be produced they could act on all the same downstream targets as the endogenous hormones. It may even be possible to replicate physiological satiety by using a mixture of agents which could mimic the panel of hormones released in response to a meal.

2.1.1 GLP-1 receptor agonists as treatments for obesity

GLP-1R agonists are currently used as a treatment for type 2 diabetes and the weight loss associated with these agents makes them particularly appropriate for obese patients. Small molecule agonists of class B GPCRs, such as the GLP-1R, have proven elusive, although some have been identified (Knudsen et al., 2007; Sloop et al., 2010), and thus peptide agonists have been a major focus for research. As GLP-1 itself is rapidly degraded by dipeptidyl peptidase IV, which is present in plasma (Deacon et al., 1995), degradation resistant analogues have been developed. Notable amongst these is exendin-4 (EX-4), a 39 amino-acid peptide with 53% homology to GLP-1 (Figure 2.1), which occurs naturally in the venom of the Gila monster Heloderma suspectum. EX-4 has been licensed for treatment of type 2 diabetes since 2005 and in addition to its beneficial effects on insulin release, EX-4 has also been shown to cause weight loss (Buse et al., 2004; DeFronzo et al., 2005; Kendall et al., 2005). Liraglutide is an analogue of GLP-1 with 97% homology to the native peptide (Figure 2.1), with the addition of a fatty acid side chain which allows it to bind to albumin giving it a half life of almost 13 hours (Agerso et al., 2002). Liraglutide was shown to be efficacious in a double blind placebo
controlled trial looking at weight loss and appeared superior to orlistat (Astrup et al., 2009). Nausea and vomiting were observed in individuals treated with liraglutide in this study. These adverse effects are likely to limit the dose, and thus the weight loss that can be achieved by liraglutide treatment. Combination of GLP-1R agonists with weight reducing agents may therefore be required to achieve significant weight loss.
Figure 2.1: A: Amino acid structures of GLP-1, and the GLP-1R agonists EX-4 and Liraglutide. B: Amino acid structures of GLP-1, Glucagon and Oxyntomodulin. Green indicates amino acids common to GLP-1 and glucagon, pink indicates amino acids seen in GLP-1 but not glucagon, blue indicates amino acids in glucagon but not GLP-1. Red indicates amino acid substitutions seen in EX-4 when compared to GLP-1. Orange shows amino acid substitutions seen in liraglutide when compared to GLP-1 (with the highlighted Lys molecule being where the fatty acid chain is attached). Purple indicates the amino acid chain unique to oxyntomodulin in this family of peptides.
2.1.1.1 GLP-1/glucagon receptor co-agonists

Although GLP-1R agonists continue to be developed with the aim of treating both Type 2 diabetes and obesity, a new class of dual agonists at both the GLP-1 and glucagon receptors are also in development. These are inspired by the endogenous GLP-1/glucagon co-agonist oxyntomodulin, and aim to combine the anorectic effects of GLP-1 with glucagon-induced increased energy expenditure (see 1.4.2.4.3).

The hyperglycemic effect of glucagon is a potential concern if glucagon receptor agonists are to be used to treat obesity, particularly as this population are at risk for type 2 diabetes. This concern is supported by studies in glucagon receptor knockout mice which have improved glucose tolerance compared to wild type mice (Parker et al., 2002) and appear to have some resistance to diet induced obesity (Conarello et al., 2007). In fact, glucagon receptor antagonists have been proposed and investigated as a treatment for type 2 diabetes for 30 years, although none have come to market (Johnson et al., 1982; Gysin et al., 1987; Unson et al., 1989; Gu et al., 2010; Mu et al., 2011). It is hoped that a dual GLP-1/glucagon receptor agonist may be able to overcome the hyperglycaemic effect of glucagon receptor activation due to the incretin effect of GLP-1. For this reason the ratio of GLP-1 to glucagon receptor activation in a dual agonist is thought to be of importance in preventing hyperglycemia (Day et al., 2009).

2.1.1.1.1 Oxyntomodulin analogues as therapeutics

An oxyntomodulin analogue has been developed which has been shown to reduce food intake and body weight in rats (Liu et al., 2010) and is currently in phase I trials for treatment of obesity. A pegylated oxyntomodulin molecule has also been produced which appeared promising in preclinical trials (Kerr et al., 2010). Oxyntomodulin does not appear to cause hyperglycemia in rodents, where acute administration actually increases glucose tolerance, suggesting that, when accompanied by GLP-1R activation, glucagon receptor activation does not necessarily produce hyperglycemia (Parlevliet et al., 2008). To achieve the optimal ratio of GLP-1 to glucagon receptor activity it may be
advantageous to use co-administration of sole GLP-1 and glucagon receptor agonists such that the ratios can be easily adjusted.

2.1.1.1.2 Novel GLP-1/glucagon receptor co-agonists

In 2009 two independent groups released the results of pre-clinical studies using their GLP-1/Glucagon co-agonists. Pocai et al., showed their dual agonist (DualAG), a cholesterol conjugated oxyntomodulin analogue, to be superior at reducing food intake and body weight in a diet induced obese (DIO) mouse model compared to a structurally similar molecule with no affinity for the glucagon receptor (Pocai et al., 2009). Day et al., synthesised a series of pegylated chimeric molecules based around the N terminal residues of glucagon and the C terminus of GLP-1 which also reduced food intake and body weight in DIO mice (Day et al., 2009). Like oxyntomodulin, the dual agonists used in these studies both increased energy expenditure as well as reducing food intake.
2.1.2 Our strategy

Our laboratory aims to produce two agents, one a GLP-1 receptor agonist and one a glucagon receptor agonist, to be given in combination with the goal of reducing food intake and increasing energy expenditure. To this end the following properties, amongst others, are important;

1. The agents must be agonists at the human GLP-1 and glucagon receptors respectively
2. The GLP-1R agonist must reduce food intake
3. The glucagon receptor agonist must increase energy expenditure when administered chronically
4. The combined administration of the GLP-1 and glucagon agonists must lead to a reduction in body weight (≥5%) which is sustained while the treatment is continued
5. The duration of action must be long enough to match or exceed that of similar agents currently available
6. There must be an improvement in glucose tolerance when the two agents are administered chronically in combination
7. A significant reduction in body weight (≥5%) should be achievable without inducing significant aversion

This chapter will aim to address points 1-6.
2.2 **CHAPTER SUMMARY AND AIMS**

The experiments contained within this chapter aim firstly to examine, in mice, the anorexigenic effects of glucagon and GLP-1, both individually and in combination. Subsequently the effects of two long lasting analogues of GLP-1 (GLPAg) and glucagon (GCGAg) on feeding were examined as part of a drug development programme taking place in this laboratory. The chronic effects of these agonists were also examined, with the aim of determining whether long acting analogues of GLP-1 and glucagon can have effects on food intake and energy expenditure leading to weight loss. Finally the effects of chronic administration of these analogues on carbohydrate tolerance in an obese mouse model were also examined.
2.3 MATERIALS AND METHODS

2.3.1 Receptor Binding Assays (RBA)

Receptor binding assays were carried out as part of a high throughput screening process, to select peptides with high affinity for the GLP-1 or glucagon receptor. The receptor binding was compared across human, mouse and rat receptors, as whilst the aim of the project is to produce a peptide with likely efficacy in humans, preclinical testing in mice and rats requires the peptides to also bind to the receptors in these species.

2.3.1.1 Radioligand production

Radiolabelled GLP-1 and glucagon were produced by direct iodination (Owji et al., 1995). 15 μg of peptide was added to 37 MBq of Na\textsuperscript{125}I (Amersham-Pharmacia Biotech, Buckinghamshire, UK) and 10 μg of 1,3,4,6,-tetrachloro-3α, 6α-diphenylglycouril (Iodogen reagent, Pierce Chemical Co, Rockford, IL, USA) for 4 minutes at 22°C. The iodinated peptide was purified by reverse phase high performance liquid chromatography using ACN/H\textsubscript{2}O gradient.

2.3.1.2 Preparation of membrane from cell lines expressing GLP-1 R or GCGR

HEK 293 cells stably overexpressing the human GLP-1 or glucagon receptor were cultured in Dulbecco’s modified medium (DMEM) without sodium pyruvate, containing 4.5 g/l glucose (Invitrogen), supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% Penicillin/Streptomycin (Invitrogen). Medium was changed every 2-3 days and cells passaged when 70% confluent using a non-enzymatic cell dissociation buffer (Sigma-Aldrich) and transferred to new flasks at a 1:5 dilution.

Each membrane preparation used a minimum of 30 T 175cm\textsuperscript{2} flasks of 70-90% confluent cells. Culture medium was removed from the flasks and cells detached by scraping in ice cold 0.02M PBS. Cells were centrifuged at 200g for 5 minutes (Sigma Centrifuges 3-18K). The supernatants were discarded and the cell pellets put onto ice before being resuspended in 20 ml ice cold homogenation buffer and homogenised for 1 minute (Ultra Turrax homogeniser, IKA Labortechnik, Staufen,
Germany). Homogenates were centrifuged for 30 minutes at 4°C at 100000g (Sorvall OTD 55B ultra-centrifuge, DuPont). Supernatants were discarded and pellets resuspended in homogenisation buffer without sucrose using a hand-held/Teflon homogeniser (Jencons, Leighton Buzzard, UK). Pellets were resuspended to a protein concentration of 1-2 mg/ml and stored at -80°C.

2.3.1.3 Preparation of membrane from mouse and rat tissue

Animals were killed by asphyxiation in a rising concentration of CO₂. Organs for membrane preparation were immediately removed and snap frozen in liquid nitrogen before being stored at -80°C. Tissues were homogenised (Ultra Turrax homogeniser (IKA Labortechnik, Staufen, Germany)) in ice cold homogenisation buffer at 4°C. Homogenates were centrifuged at 1000g (Beckman J2-21, rotor JS-13.1) for 15 minutes at 4°C, and supernatants were then centrifuged at 100000g for 1 hour at 4°C (Sorvall OTD 55B ultra-centrifuge, DuPont). Pellets were removed and resuspended in 10 volumes of the homogenation buffer without sucrose and centrifuged at 100000g for 1 hour at 4°C. Pellets were resuspended in homogenation buffer without sucrose to a final protein concentration of 2-3 mg/ml and stored at -80°C.

2.3.1.4 Receptor Binding Assay

Membranes prepared as described above were incubated for 90 minutes at room temperature in 1.5 ml siliconised microtubes (Sigma-Aldrich) in GLP-1 or glucagon RBA buffer (4°C) with ¹²⁵I-GLP-1 or ¹²⁵I-glucagon. Each tube contained 1000 counts/sec of radioligand in a final volume of 0.5 ml. Unlabelled peptide was added at increasing concentrations to the tubes in duplicate or triplicate. After a 90 minute incubation period the membrane (containing bound peptide) was separated from the unbound peptide by centrifugation at 4°C at 15600 g (Sigma Centrifuges, 3-18K) for 3 minutes and the supernatant discarded. The pellet was resuspended in 0.5 ml of the appropriate RBA buffer and the centrifugation repeated. The supernatant was once again discarded and the bound radiolabelled peptide was measured using a γ-counter (NE 1600, NE Technology Ltd.). Specific binding of the labelled peptide was expressed as the difference between the counts measured in the
absence and presence of unlabelled peptide. IC\textsubscript{50} values were calculated using Prism (Version 5.00) (GraphPad Software Inc.).

2.3.2 In vivo studies

2.3.2.1 Animals

All animal procedures undertaken were approved by the British Home Office under the UK Animal (Scientific Procedures) Act 1986 (Project license 70/7236). Male C57Bl/6 mice (Harlan, UK) or male Wistar Rats (Charles River, UK) were housed in individually ventilated cages under controlled temperature (21-23°C) and a 12:12 hour light-dark cycle (lights on 0700). Animals had ad libitum access to food (RM1 diet, Special Diet Services Ltd, Witham, UK) and water unless otherwise stated. Animals were handled regularly and acclimatised to subcutaneous (s/c) injections prior to the studies.

2.3.2.2 Peptides

All peptides purchased from Bachem (St Helens, UK) and stored at -20°C. GLP\textsubscript{Ag} and GCG\textsubscript{Ag} were designed by Professor Stephen Bloom. Their sequences are based on those of GLP-1 and Glucagon respectively. Amino acid substitutions were made in sites known to be important for the enzymatic degradation of these hormones, such as the cleavage between amino acids 2 and 3 by DPPIV, to reduce the activity of these enzymes. Other substitutions were made to increase the binding affinity of the peptides, such as incorporating elements of the sequence of EX-4 into that of GLP\textsubscript{Ag}. Peptides were dissolved in 0.9% w/v sterile saline for in vivo experiments.

2.3.2.3 Acute Feeding studies

The effect of candidate peptides on feeding in mice over a 24 hour period was assessed as the primary indicator of bioactivity for peptides which bind to the GLP-1R. Glucagon receptor agonists were also tested, although their efficacy was primarily assessed in a pair-feeding paradigm (section 2.3.2.5).
2.3.2.3.1 Animals
Mice weighed 20-30g (weight range and means are given for individual studies) and were singly housed.

2.3.2.3.2 Procedure
Fasted mice were used as both glucagon and GLP-1 have previously been shown to be anorectic and these effects are more easily demonstrated in a fasted paradigm. Mice were fasted overnight (from 1600) prior to the experiments. Experiments began in the early light phase (0900). Mice were randomised by body weight into groups. A saline control group (vehicle) was used in all studies. 50μl injections were given s/c and a time zero food weight was recorded. Balances were accurate to 0.01g (Sartorius, UK). The remaining food was reweighed at intervals specified in each individual study. During reweighs a visual inspection was made of the cage to check for spillage of food from the hopper.

2.3.2.3.3 Combined GLP-1 and glucagon feeding study procedure
This experiment aimed to test the hypothesis that a combination of glucagon and GLP-1 would be more anorectic than either peptide given individually. Peptide doses for this study were chosen based on the dose response studies (Figure 2.6, Figure 2.7). High doses (750 nmol/kg glucagon, 300 nmol/kg GLP-1) aimed to cause 80% inhibition of food intake, medium doses (100 nmol/kg glucagon, 30 nmol/kg GLP-1) aimed to reduce food intake to 50% of that of the saline control group and low doses (30 nmol/kg glucagon, 10 nmol/kg GLP-1) aimed to be the highest doses that did not significantly inhibit food intake. Each mouse received two injections within 10 seconds. Mice only receiving one peptide received a second saline injection.

2.3.2.4 Chronic feeding study in Diet Induced Obese (DIO) mice
This experiment aimed to test the weight loss induced by daily injections of the candidate peptides GLPAg and GCGAg, individually and in combination, in an obese model. In particular, as DIO mice have impaired glucose tolerance, this model was used to assess the ability of these peptide
treatments to improve the response to a glucose tolerance test. Low doses of GLPAg and GCGAg were selected such that the weight loss induced by the agents individually would not be so great as to prevent any additional effect of a combination being detectable.

2.3.2.4.1 Animals
Mice were fed on a high fat diet (60% kcal fat) (Research Diets, New Brunswick, New Jersey) and the study began when mice were at a mean weight of 38g. Animals were handled regularly and acclimatised to subcutaneous (s/c) injections prior to the studies.

2.3.2.4.2 Procedure
Mice were randomised by body weight into groups. 50 μl s/c injections were given at 1700h on a daily basis throughout the study. Food and body weight were recorded at the same time for the first 28 days of the study and at 3 times per week thereafter. Doses of GCGAg and GLPAg were doubled from 20 to 40nmol/kg and 5 to 10 nmol/kg respectively after 6 days, and the dose of GCGAg was doubled again from day 16 onwards from 40 nmol/kg to 80 nmol/kg. These dose escalations were necessary as these peptides had not previously been tested on a chronic basis in DIO mice, and thus accurate predictions of the effect sizes were not possible. In addition, dose escalation protocols are often used in humans when EX-4 treatment is initiated as a treatment for type 2 diabetes, and has been shown to reduce the side effects experienced by patients (Fineman et al., 2004).

2.3.2.4.3 Glucose Tolerance Test
A glucose tolerance test (GTT) was carried out in DIO mice after the 70 day chronic study. Mice were injected on the morning of the study (0700) with the same treatment they had received throughout. This injection was given such that the effects of the peptides on glucose tolerance could be observed over a period in which they could still be expected to be present in the circulation. Animals were then fasted for 6 hours prior to glucose injection, and for the duration of the study. Glucose was injected intraperitoneally at a dose of 2 g/kg in a maximum volume of 0.5 ml at time 0. At t=-30 tail tipping was performed and a small sample of blood used to measure baseline blood glucose using a
glucometer (CONTOUR meter and test strips, Bayer, Berkshire, UK). Glucose was measured at t=-30, 0, 30, 60, 90 and 120 minutes. Approximately 20 μl of blood for measurement of baseline insulin was collected in Lithium-Heparin microvettes (Starstedt, Leicester, UK) and kept on ice. Blood was taken to measure insulin at t=0, 30, 60 and 120 minutes. Mice were returned to their home cages between all measurements.

Incremental area under the curve was calculated as the total area under the blood glucose curve for each individual animal, from which an area equal to the baseline blood glucose for that animal multiplied by the length of the test (120 minutes) was subtracted.

2.3.2.4.3.1 Measurement of plasma insulin

Blood samples were stored on ice for up to one hour before being centrifuged at 10 000 rpm at 4°C (Sigma Centrifuges 3-18K) The plasma supernatant was removed and stored at -20°C for no more than 2 weeks before being assayed for insulin content. Insulin was measured using a mouse insulin ELISA kit as described in the assay manual (Mouse Ultra Sensitive Insulin ELISA kit, Crystal Chem, Illinois, USA). Briefly, samples and insulin standards are incubated in a plate pre-coated with guinea pig anti-mouse insulin antibody then subsequently with a horse radish peroxidise-conjugated anti-insulin antibody which binds to the existing antibody-antigen complexes. Finally a 3, 3', 5, 5' tetramethylbenzidine substrate solution is added to detect the presence of the horse radish peroxidise enzyme. Absorbance is measured at A450 and A630 (Multiskan RC 381, Labsystems, MA, USA) and the A630 measurement is subtracted from the A450 measurement. The insulin concentrations of unknown samples are interpolated using a standard curve from insulin standards prepared as described in the assay manual (GraphPad, Prism).

2.3.2.5 Pair-feeding Studies

Pair-feeding studies were used to give an indication of the effects of a peptide on energy expenditure. As well as comparing the body weight and food intake of a peptide treated group with that of a saline control group, an additional saline treated control group are restricted to consuming
the median weight of food consumed by the peptide treated animals in each 24 hour period. Thus as the peptide-treated and pair-fed groups have the same food intake, any difference in body weight is more likely to be due to an effect of the peptide on energy expenditure.

2.3.2.5.1 Animals

Rats (Charles River, UK) weighed 430-595g (weight range and means are given for individual studies) and were singly housed.

2.3.2.5.2 Procedure

Rats were randomised into groups (n=6-8) stratified by body weight. Animals were injected daily for 7 days (50 μl volume) at 1700h with 50 μl of either saline or peptide and food and body weights were recorded. Where two peptides were administered this was done in a single injection. Pair-fed control groups began the study 24 hours after the saline control and peptide groups and were given only the median weight of food consumed in the previous 24 by the peptide groups they were paired to.

2.3.2.6 Pharmacokinetic studies

These studies were carried out to assess the profile of release and clearance of the peptides tested when administered subcutaneously. Male Wistar rats were group housed and given ad libitum access to food and water throughout the study. Animals were randomised by body weight into groups of 4 and each administered with 1 mg of the specified peptides at time 0. At 0 hours prior to injection and at 4, 24, 48 and 72 hours following administration, blood was collected by superficial venesection of the lateral tail vein. Blood was collected into eppendorfs which had been flushed with a solution containing 1:10 heparin:saline. 100-200 μl samples of blood were taken at each time point and kept on ice. Samples were centrifuged for at 4°C for 10 minutes at 7000 x g (Sigma Centrifuges 3-18K) and the plasma layer was removed and stored at -20°C to be assayed by radioimmunoassay.
2.3.2.6.1 Radioimmunoassays

All radioimmunoassays (RIAs) used were derived and maintained by Professor MA Ghatei (Professor of Regulatory Peptides, Metabolic Medicine, Faculty of Medicine, Imperial College). All reagents and materials other than peptides were supplied by Sigma. The principles of RIAs are explained in section 6.1. The antibody used for these assays was raised in rabbits against a peptide fragment consisting of the N terminal 16 amino acids of the human glucagon sequence. Lower limits of detection were calculated as specified in (Bloom and Long, 1982). Values below the lower limit of detection are plotted as zero on the graphs.
2.3.3 Statistics

Results are expressed as mean ± standard error of the mean (SEM) for all studies. In acute feeding studies the unpaired two tailed T Test was used for comparisons between two groups or one-way analysis of variance (ANOVA) was used for comparisons between three or more groups at individual time points. A post hoc Dunnett’s test was used to compare each group to a saline control in dose response studies. Bonferroni’s multiple comparison test was used in acute co-administration studies to compare pre-selected pairs of treatments. For comparing baseline glucose or insulin values and area under the curve values for the GTT, a one way ANOVA was used followed by Tukey’s post hoc test comparing all groups to one another. Analyses were performed using Prism version 4.03 software (Graphpad Software, San Diego, CA, USA). The threshold for statistical significance was set at p<0.05.

Continuous data including cumulative food intake or body weight data in chronic feeding studies were analysed using the generalised- estimating equation (GEE), a specialised version of the generalised linear model (GLM) designed for repeated measures. This test estimates the correlation between an individual or groups response to treatment and estimates the effect’s magnitude and variance. If significant differences between two groups were found using the GEE a Mann-Whitney U test was used to compare the treatments at each individual time point. When more than one pair of groups was compared using the GEE a Bonferroni correction was applied to prevent an increase in the likelihood of type I errors. Unless otherwise stated significance markers on graphs represent a significant difference in the GEE model, and the results of the Mann Whitney U test are only shown if a significant difference does not emerge until later time points, or is apparent initially and not at later stages of the study.
2.4 RESULTS

2.4.1 Receptor Binding

2.4.1.1 Glucagon

Figure 2.2 A shows a representative graph comparing the ability of glucagon to displace $^{125}$I–labelled glucagon from the human, mouse and rat glucagon receptors. The mean IC50 for glucagon binding to the human, mouse and rat glucagon receptors were found to be 1.50 nM (±0.10), 1.3 nM (± 0.21) and 0.63 nM (± 0.05) respectively (n=3). For comparison, the IC50 for glucagon binding to the human GLP-1R was found to be in the region of three orders of magnitude higher (5 μM (± 1), (Figure 2.2 B).

2.4.1.2 GLP-1

Figure 2.3 A shows a representative graph comparing the ability of GLP-1 to displace $^{125}$I–labelled GLP-1 from the human, mouse and rat GLP-1Rs. The mean IC50 for GLP-1 binding to the human, mouse and rat GLP-1Rs were found to be 0.72 nM (±0.19), 0.2 nM (± 0.01) and 0.64 nM (± 0.38) respectively (n=3). For comparison, the IC50 for GLP-1 binding to the human glucagon receptor was found to be in the region of four orders of magnitude higher (220μM (± 70), (Figure 2.3 B).
Figure 2.2: Competitive binding of $^{125}$I-glucagon and glucagon at the human, mouse and rat glucagon receptors (A) and of $^{125}$I-GLP-1 and glucagon at the human GLP-1R (B).

Figure 2.3: Competitive binding of $^{125}$I-GLP-1 and GLP-1 at the human, mouse and rat GLP-1Rs (A) and of $^{125}$I-glucagon and GLP-1 at the human glucagon receptor (B).
2.4.1.3 Exendin 4 (EX-4)

Figure 2.4 A shows a representative graph comparing the ability of EX-4 to displace $^{125}$I–labelled GLP-1 from the human, mouse and rat GLP-1Rs. The mean IC$_{50}$ for EX-4 binding to the human, mouse and rat GLP-1Rs were found to be 0.34 nM (±0.04), 0.28 nM (± 0.01) and 0.37 nM (± 0.03) respectively (n=3).

2.4.1.4 GLPAg: A GLP-1 receptor agonist

Figure 2.4 B shows a representative graph comparing the ability of GLPAg to displace $^{125}$I–labelled GLP-1 from the human, mouse and rat GLP-1Rs. The mean IC$_{50}$ for GLPAg binding to the human, mouse and rat GLP-1Rs were found to be 1.8 nM (±0.27), 2.1 nM (± 0.5) and 1.7 nM (± 0.03) respectively (n=3). GLPAg showed no binding to the human, mouse or rat glucagon receptors.

2.4.1.5 GCGAg: A glucagon receptor agonist

Figure 2.5 A shows a representative graph comparing the ability of GCGAg to displace $^{125}$I–labelled glucagon from the human, mouse and rat glucagon receptors. The mean IC$_{50}$ for GCGAg binding to the human, mouse and rat glucagon receptors were found to be 1.6 nM (±0.27), 1.0 nM (± 0.6) and 0.67 nM (± 0.3) respectively (n=3). GCGAg also binds to the GLP-1R Figure 2.5 B, although the affinity is in the region of two orders of magnitude lower than for its binding to the glucagon receptor (290 nM (±86), 91 nM (±31) and 130 nM (±44) for the human, mouse and rat GLP-1R respectively).
Figure 2.4: A Competitive binding of $^{125}$I-GLP-1 and EX-4 or GLPAg at the human, mouse and rat GLP-1Rs.
B Competitive binding of $^{125}$I-GLP-1 and GLPAg at the human, mouse and rat GLP-1Rs.

Figure 2.5: A Competitive binding of $^{125}$I-glucagon and GCGAg at the human, mouse and rat glucagon receptors and B, of $^{125}$I-GLP-1 and GCGAg at the human, mouse and rat GLP-1Rs.
2.4.2 Acute effects on food intake

2.4.2.1 Food intake in response to a range of glucagon doses

The dose response relationship for glucagon and food intake is shown in Figure 2.6. The lowest effective dose used in this study was 100 nmol/kg. Beyond the 30 minute time point no statistically significant effect of any dose on food intake was observed, however there was a trend towards a dose dependent rebound increase in food intake at 30-90 minutes which was delayed until the 90-180 minute timepoint in the 750 nmol/kg group.

Figure 2.6: Effect of a range of doses of glucagon on food intake. A: 0-30 minutes, B: 30-90 minutes, C: 90-180 minutes, D: cumulative food intake. All doses in nmol/kg bodyweight, mean mouse weight 31.5 g. Mice were fasted overnight, injected at the onset of the light phase s/c and given a measured amount of food. Food was reweighed at the specified times. N=5 *p<0.05, **p<0.01, ***p<0.001
2.4.2.2 Food intake in response to a range of GLP-1 doses

The dose response relationship for GLP-1 and food intake is shown in Figure 2.7. The lowest effective dose was 30 nmol/kg. In the 30-90 minute interval, only 600 nmol/kg reduced food intake significantly and no reduction of food intake occurred with any dose at the 90-180 minute interval.

Figure 2.7: Effect of a range of GLP-1 doses on food intake in fasted mice. A 0-30 minutes, B 30-90 minutes, C 90-180 minutes, D 0-180 minutes. Doses in nmol/kg bodyweight, mean mouse weight 31.5 g. Mice were fasted overnight, injected at the onset of the light phase s/c and given a measured amount of food. Food was reweighed at the specified times. N=5.*p<0.05, **p<0.01, ***p<0.001
2.4.2.3 Acute food intake in response to GLPAg

Food intake was measured at intervals over a 24 hour period in response to subcutaneous injection of GLPAg (50 nmol/kg) in mice (Figure 2.8). At all time points measured between 1 and 24 hours there was a statistically significant difference in cumulative food intake between saline and GLPAg treated mice. Further to this there was a statistically significant difference in interval food intake at the 0-1, 1-2 and 2-4 hour timepoints but not at the later intervals measured.
Figure 2.8: Effect of GLPAg on food intake over 24 hours. Mice were fasted overnight, injected at the onset of the light phase subcutaneously with either saline or GLPAg (50nmol/kg) and given a measured amount of food. Food was reweighed at the specified times. Mean mouse weight 30.5 g A: cumulative food intake, B-F: interval food intake N=6. *p<0.05, **p<0.01, ***p<0.001
2.4.2.4 Acute food intake in response to GCGAg

Food intake was measured at intervals over a 24 hour period in response to subcutaneous injection of GCGAg (50 nmol/kg) in mice (Figure 2.9). Mean weight of mice in this study was 32.0 g. There was no statistically significant difference in cumulative food intake between saline and GCGAg treated mice. However there was a statistically significant reduction in food intake at the 0-1 hour interval and a significant increase in food intake at the 1-2 hour interval in response to GCGAg.
Figure 2.9: Effect of GCGAg on food intake over 24 hours. Mice were fasted overnight, injected at the onset of the light phase subcutaneously with either saline or GCGAg (50nmol/kg) and given a measured amount of food. Food was reweighed at the specified times. Mean mouse weight 32.0 g A: cumulative food intake, B-F: interval food intake N=6. *p<0.05, **p<0.01, ***p<0.001
2.4.2.5 The effects of glucagon and GLP-1 individually and in combination

Figure 2.10 shows the effects of simultaneous co-administration of GLP-1 and glucagon at three doses compared with the effects of these peptides alone. Mice in this study weighed an average of 31.8 g. Three doses of each peptide were chosen based on results of individual hormone dose responses (Figure 2.6 and Figure 2.7). At high (300 nmol/kg GLP-1, 750 nmol/kg glucagon) and medium (30 nmol/kg GLP-1, 100 nmol/kg glucagon) and low (10 nmol/kg GLP-1, 30 nmol/kg glucagon) doses, the combination of GLP-1 and glucagon appeared to cause a greater reduction in food intake than either peptide alone at the same dose. This effect was statistically significant between the low dose glucagon and low dose GLP-1/glucagon group. The effect of these peptides had worn off by the 30-90 time point. It should be noted the rebound food intake at the 30-90 minute time point was larger in the medium dose combined GLP-1/glucagon group than in the medium dose GLP-1 alone group.
Figure 2.10: Effect of GLP-1 and glucagon at a range of doses and in combination on food intake. A 0-30 minutes, B 30-90 minutes, C 90-180 minutes. Does in nmol/kg bodyweight, mean mouse weight 31.8 g. Mice were fasted overnight, injected at the onset of the light phase s/c and given a measured amount of food. Food was reweighed at the specified times. N=6. *p<0.05, **p<0.01, ***p<0.001
2.4.3 Pharmacokinetic studies in rats

2.4.3.1 GLP\(_\text{Ag}\)

Plasma levels of GLP\(_\text{Ag}\) were measured using an RIA with an antibody raised against the first 16 amino acids of the glucagon sequence (Figure 2.11). The peak concentration was seen at 4 hours post injection, after which time levels of GLP\(_\text{Ag}\) fell, but had still not returned to baseline at 72 hours.

![Plasma levels of GLP\(_\text{Ag}\)](image)

**Figure 2.11**: Pharmacokinetics of subcutaneously administered GLP\(_\text{Ag}\) in rats. 1mg GLP\(_\text{Ag}\) administered subcutaneously at time 0. Blood samples from the tail vein taken at the specified times and assayed for GLP\(_\text{Ag}\) by RIA. Lower limit of detection 365 pmol/L (dashed line) N=4.
2.4.3.2 GCGAg

Plasma levels of GCGAg were measured using an RIA for which the antibody was raised against with an antibody raised against the first 16 amino acids of the glucagon sequence (Figure 2.12). The peak concentration was seen 4 hours post injection and had not returned to baseline 72 hours after injection.

![Graph showing plasma levels of GCGAg over time](image)

**Figure 2.12: Pharmacokinetics of subcutaneously administered GCGAg in rats.** 1mg GCGAg administered subcutaneously at time 0. Blood samples from the tail vein taken at the specified times and assayed for by RIA. Lower limit of detection 895 pmol/L (dashed line) N=4.
2.4.4 Effect of chronic peptide administration on food intake and body weight in rats

2.4.4.1 EX-4

To screen potential peptides to be used as glucagon receptor agonists in combination with a GLP-1R agonist, candidate peptides were administered chronically to rats in combination with EX-4. The effects of EX-4 alone on food intake and body weight were therefore studied.

Food intake and bodyweight were monitored in response to daily administration of EX-4 (5 nmol/kg) in rats. Cumulative food intake was significantly different in the EX-4 and EX-4 pair-fed groups to saline treated animals between days 1-7, and there was no significant difference between the EX-4 and EX-4 pair-fed group (Figure 2.13 A). The difference in cumulative food intake was reflected in a difference in daily food intake that was maintained throughout the 7 day period (Figure 2.13 C).

Whilst there was no statistically significant difference in body weight between any of the groups, there were statistically significant differences between the body weight change seen in both the EX-4 and EX-4 pair-fed groups compared to the saline controls Figure 2.13 B. There was no difference between EX-4 treated and EX-4 pair-fed groups.
Figure 2.13: Effect of daily EX-4 for 7 days on food intake and body weight in rats. Rats were *ad libitum* fed (with the exception of the pair fed group) and injected s/c in the late light phase. Average starting body weight 368 g. Body weight and food intake were also measured at the time of injection. Saline treated animals shown in black, EX-4 (5 nmol/kg) treated in red solid line, animals pair-fed (PF) to EX-4 treated shown in red dashed lines. N=8-9. *p<0.05, ** p<0.01 ***p<0.001 comparing EX-4 to saline $p<0.05, \circ p<0.01 \circ\circ p<0.001$ comparing pair fed to saline. A: Cumulative food intake B: Body weight change C: Daily food intake D: Absolute body weight.
2.4.4.2 GCGAg

The purpose of this study was to determine whether GCGAg had an effect on body weight when administered once daily, and whether it altered energy expenditure using a pair-feeding paradigm.

Food intake was monitored in response to daily administration of EX-4 (5 nmol/kg), GCGAg (100 nmol/kg) and GCGAg (100 nmol/kg) + EX-4 (5 nmol/kg) in rats. Cumulative food intake was significantly different between EX-4 and saline treated animals (Figure 2.14 A). The combination of GCGAg and EX-4 reduced cumulative food intake compared to saline and compared to EX-4 alone. Daily food intake was also significantly different in both the EX-4 and GCGAg + EX-4 groups compared to saline controls across the 7 day study, and there was a significant difference in daily food intake between the EX-4 and EX-4 + GCGAg groups (Figure 2.14 C).

Absolute body weight was not found to be significantly different between any of groups, although a trend towards reduced body weight in the GCGAg + EX-4 group compared to controls was seen (p=0.052) (Figure 2.14 D). However the body weight change from initial was significantly different in all groups compared to saline controls (Figure 2.14 B). The GCGAg + EX-4 group lost significantly more weight than the group treated with EX-4 alone. In addition the GCGAg + EX-4 treated group lost more weight than their pair-fed control group.
Figure 2.14: Effect of daily GCGAg for 7 days on food intake and body weight in rats. Rats were *ad libitum* fed and injected s/c in the late light phase. Mean starting body weight 501 g. Body weight and food intake were also measured at the time of injection. Saline treated animals shown in black, EX-4 (5 nmol/kg) treated in red solid line, GCGAg (100 nmol/kg) + EX-4 (5 nmol/kg) treated shown in purple solid line and GCGAg + EX-4 pair-fed group shown in purple dashed lines. N=6. *p<0.05, ** p<0.01 ***p<0.001 comparing EX-4 to saline , in place of * are ′ comparing GCGAg+EX-4 to saline, ″ comparing GCGAg+EX-4 pair-fed to saline, ′ comparing EX-4 to GCGAg+ EX-4 and ″ comparing GCGAg+EX-4 to their pair-fed controls. A: Cumulative food intake B: Body weight change C: Daily food intake D: Absolute body weight.
2.4.5 Effect of chronic administration of GLPAg and GCGAg alone and in combination in DIO mice

2.4.5.1 Food intake and body weight

Food intake was monitored in response to daily administration of GLPAg, GCGAg or GLPAg + GCGAg in diet induced obese mice (Figure 2.15 A). Cumulative food intake was not reduced in any of the treatment groups compared to saline controls.

Absolute body weight was not found to be significantly different between any of the groups (Figure 2.16 A). The body weight change was however significantly different from saline controls in the GLPAg treated and GLPAg + GCGAg treated groups (Figure 2.16 B). There was no significant difference in body weight change between the GLPAg alone and GLPAg + GCGAg groups.

The percentage body weight changes (normalised to saline controls) are shown in Figure 2.16 C. The group receiving a combination of GLPAg and GCGAg had a mean reduction of 9.6 % of their body weight when compared to the change in body weight seen in saline controls over the 70 day period. In contrast the GLPAg and GCGAg groups had 2.9 % and 1.8 % comparative reductions respectively.
Figure 2.15: Effect of daily GLPAg, GCGAg or GLPAg+GCGAg for 70 days on food intake in diet induced obese mice. Mice were fed ad libitum on a high fat diet and injected daily s/c at 17:00. Body weight and food intake were measured at the time of injection. Mean starting body weight 38.1 g. Saline treated animals shown in black, GLPAg treated in red, GCGAg treated in blue and GLPAg + GCGAg treated in purple. Dose of GLPAg 5nmol/kg days 0-5 and 10nmol/kg thereafter, Dose of GCGAg 20nmol/k days 0-5, 40nmol/kg days 6-15 and 80nmol/kg thereafter. N=10-12. *p<0.05, ** p<0.01 ***p<0.001, in place of * are $\$ comparing GCGAg to saline, and $#$ comparing GLPAg + GCGAg to saline, A: Cumulative Food intake B: 24 hour food intake.
Figure 2.16: Effect of daily GLPAg, GCGAg or GLPAg+GCGAg for 70 days on body weight in diet induced obese mice. Mice were fed ad libitum on a high fat diet and injected daily s/c at 17:00. Body weight and food intake were measured at the time of injection. Mean starting body weight 38.1 g. A: Absolute body weights, B: Body weight change, C: Body weight change as a percentage corrected to saline controls. Saline treated animals shown in black, GLPAg treated in red, GCGAg treated in blue and GLPAg +GCGAg treated in purple. Dose of GLPAg 5nmol/kg days 0-5 and 10nmol/kg thereafter, Dose of GCGAg 20nmol/kg days 0-5, 40nmol/kg days 6-15 and 80nmol/kg thereafter. N=10-12. *p<0.05, ** p<0.01 ***p<0.001. * comparing GLPAg to saline, # comparing GLPAg+GCGAg to saline.
2.4.5.2 Intraperitoneal glucose tolerance test

Figure 2.17 A and B show the response of blood glucose and serum insulin respectively to an IP glucose load after 70 days of the specified treatments. At baseline, after a 5 hour daytime fast, all the treatment groups had significantly lower blood glucose than saline controls (Figure 2.17 C). The GLPAg + GCGAg group had significantly lower blood glucose at baseline than either of the groups given these peptides individually. At baseline the GCGAg and GLPAg + GCGAg groups had lower serum insulin levels than the saline control animals (Figure 2.17 D).

The incremental area under the curve during a glucose tolerance test is used as a measure of the change in blood glucose or insulin over the entire period of the test. All three treatment groups had significantly lower values for area under the curve of blood glucose against time than those in the saline control group (Figure 2.17 E). There was no significant difference between any of the treatment groups.

The GLPAg + GCGAg group had a significantly higher value for incremental area under the curve of serum insulin against time than those in the saline control group (Figure 2.17 F).
Figure 2.17: Effect of daily GLPAg, GCGAg or GLPAg+GCGAg for 70 days on response to glucose tolerance test. Mice were fed ad libitum on a high fat diet and injected daily s/c at 17:00. On day 70 mice were injected with 2 g/kg glucose IP at time 0. A: Blood glucose, B: Serum insulin, C: baseline blood glucose, D: Baseline serum insulin, E: Incremental area under the curve for blood glucose F: Incremental area under the curve for serum insulin. Saline treated animals shown in black, GLPAg treated in red, GCGAg treated in blue and GLPAg + GCGAg treated in purple. Dose of GLPAg 5 nmol/kg days 0-5 and 10 nmol/kg thereafter, Dose of GCGAg 20 nmol/kg days 0-5, 40 nmol/kg days 6-15 and 80 nmol/kg thereafter. N=10-12. *p<0.05, ** p<0.01 ***p<0.001
2.5 Discussion

Glucagon and GLP-1 are anorectic peptides and synthetic agonists of their receptors are being investigated as treatments for obesity and type 2 diabetes. This chapter examined the anorectic effects of glucagon and GLP-1 individually and in combination. The receptor binding and in vivo efficacy of two agents designed in this laboratory, GLP-1Ag and GCGAg were also assessed.

2.5.1 Action of glucagon

The results of acute feeding studies show that glucagon inhibited food intake in a dose dependent manner in overnight fasted mice, but not beyond 30 minutes after injection. Although there are no published reports of the effect of glucagon on acute food intake in mice, a number of studies have measured this effect in rats (Table 2.1).

<table>
<thead>
<tr>
<th>Publication</th>
<th>Dose (nmol/kg)</th>
<th>Food intake as % of control</th>
<th>Route</th>
<th>Fasting and timing of experiment</th>
<th>Protocol information</th>
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<tr>
<td>(Geary and Smith, 1982b)</td>
<td>7</td>
<td>45.5 **</td>
<td>IP</td>
<td>Ad libitum fed, early light phase</td>
<td>Milk diet</td>
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<tr>
<td></td>
<td>29</td>
<td>36 **</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>115</td>
<td>56 **</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Geary and Smith, 1982a)</td>
<td>29</td>
<td>90 *</td>
<td>IP</td>
<td>3 hour fast, mid light phase</td>
<td>Implanted closed gastric cannulas, stomachs drained prior to test, milk diet.</td>
</tr>
<tr>
<td></td>
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<td>80 *</td>
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<td></td>
<td>718</td>
<td>65 *</td>
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</tr>
<tr>
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<td>57</td>
<td>80 *</td>
<td>IP</td>
<td>6 hour fast, early dark phase</td>
<td>Sham AP/NTS lesion</td>
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<td>52 **</td>
<td>HPV</td>
<td>Ad libitum fed, Last quarter of dark phase</td>
<td>Hepatic portal indwelling cannula</td>
</tr>
<tr>
<td>(Geary, 1996)</td>
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<td>79 **</td>
<td>HPV</td>
<td>Ad libitum fed, Last quarter of dark phase</td>
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<td>IP</td>
<td>24h fasted, onset dark phase</td>
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<td>(Geary et al., 1997)</td>
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<td>71 **</td>
<td>HPV</td>
<td>Non-fasted, Onset of dark phase</td>
<td>Hepatic portal indwelling cannula</td>
</tr>
</tbody>
</table>

Table 2.1: Summary of published studies where glucagon was administered to rats and acute food intake measured. *Food intake at 30 minutes post injection ** first spontaneous meal size post injection. IP: intraperitoneal, HPV: hepatic portal vein
The lowest effective anorectic dose reported in rats was 7 nmol/kg (Geary and Smith, 1982b). In the current study in mice, 100 nmol/kg was the lowest effective dose tested. Whilst this disparity could be due to the difference in species, it could also be a result of difference in protocol. Geary and Smith measured spontaneous meal size which is likely to be more sensitive a measure as it is not affected by compensatory increases in food intake once the anorectic effect has worn off. The anorectic effect of glucagon peaks between 8 and 12 minutes after intraperitoneal administration (Geary and Smith, 1982b) suggesting a compensatory increase in food intake may occur within the 30 minute interval measured in the current studies. The higher doses used in the current studies and in those of Geary and Smith had more consistent effects. A 100 nmol/kg dose of glucagon reduced food intake by 54% at the 30 minute timepoint in the current study, making it comparable to the effect of a 115nmol/kg dose on spontaneous meal size observed by Geary and Smith (1982b).

100 nmol/kg of glucagon administered subcutaneously in the current study had a greater effect than was observed in published reports after administration of intraperitoneal administration of either 144 nmol/kg (Geary and Smith, 1982a) or 115 nmol/kg (Lutz et al., 1996). The current study used an overnight fasting regimen, with food intake measured in the early light phase. In contrast Geary and Smith (1982a) used only a 3 hour fast with injections in the middle of the light phase. As rats feed mainly during the dark phase this short fast is unlikely to produce a strong drive to eat in the saline control group, thus any reduced appetite caused by glucagon administration is more difficult to detect. Lutz et al (1996) used a 24 hour fast and measured food intake at the onset of the dark phase. This should maximise the appetite of the saline control group, and thus make any reduction in appetite in the glucagon control group more obvious. As this study found only a very small effect of glucagon on appetite it might be hypothesised that the endogenous orexigenic signals were strong enough to override the effects of the glucagon administered.
2.5.1.1 Which receptor does glucagon act at to inhibit food intake?

The dose response studies show that GLP-1 is approximately three times as potent as glucagon in terms of anorectic effect. This difference in in vivo potency is much smaller than the difference in receptor affinity. The IC50 for glucagon binding to the GLP-1R was three orders of magnitude higher than that of GLP-1. The fact that only a threefold increase in dose is required to give an equivalent inhibition of food intake could indicate that the anorectic effect of glucagon is unlikely to be mediated by the GLP-1R. However receptor activation was not measured and it may be that the EC50s are closer than the IC50s for these two peptides at the GLP-1R. Furthermore, glucagon has a longer circulating half life than GLP-1: 39.5 minutes compared to 5.8 minutes in rats (Oshima et al., 1988). Thus glucagon is likely to be present at higher concentrations which could compensate for its lower affinity for the GLP-1R. The potential role of the GLP-1R in mediating the anorectic effects of glucagon is investigated further in chapter 3.

2.5.2 Effect of combined administration of GLP-1 and Glucagon

The combined administration of GLP-1 and glucagon was carried out at three sets of doses. The highest pair of doses was chosen to reduce food intake by 80%, the middle set to 50% of control, and the lowest pair of doses was chosen as the maximum dose of each peptide which did not reduce food intake (all at 0-30 minute timepoint) in preceding dose-finding studies. It is clear from the results of this study that the effectiveness of the doses was not identical to that predicted from the previous dose response studies. Despite this, it appears that the combination of GLP-1 and glucagon at all three sets of doses inhibited food intake to a greater extent than either peptide alone, although this only reached statistical significance between the medium dose GLP-1 group and the medium dose GLP-1/glucagon combined group, and between the low dose glucagon and low dose GLP-1/glucagon group. At low doses neither glucagon nor GLP-1 reduced food intake when administered individually, but significantly reduced food intake when given in combination.
The effect of combining subthreshold doses of GLP-1 and glucagon appears to have an effect on food intake which is least additive. However the definitions of additive and synergistic responses are more complicated than comparing the sum of the individual responses to the combined response (Chou, 2006) and would require fuller dose response curves and complex mathematical analysis to apply to this data set.

2.5.3 Action of GLPAg

GLPAg is a GLP-1R agonist designed in our laboratory. GLPAg bound to the GLP-1R with an IC50 of approximately 2 nM, with no apparent difference in affinity at the human mouse or rat GLP-1Rs.

Subcutaneous administration of 50 nmol/kg GLPAg reduced cumulative food intake significantly over a 24 hour period, although in terms of interval food intake, reductions were only seen up to the four hour timepoint. The pharmacokinetic study shows that GLPAg appeared to reach peak plasma concentration 4 hours after administration after which point plasma concentration declined steadily over the 72 hour period of the study.

Chronic administration of a low dose of GLPAg (10 nmol/kg) to DIO mice over 70 days resulted in no reduction in cumulative food intake compared to saline controls (Figure 2.15). The daily food intake graph (Figure 2.15 B) indicates that perhaps GLPAg reduced food intake compared to saline controls in the first few days of the study, but this did not reach significance. This lack of effect on food intake is probably due to the low dose of GLPAg used, and higher doses could be used to give an increased anorectic effect.

Despite having no effect on food intake in this chronic study, the body weight change in GLPAg treated animals was significantly different from saline controls. This could suggest an effect on energy expenditure, although published data suggests GLP-1R agonists do not increase energy expenditure (Baggio et al., 2004b; Flint et al., 2000; Knauf et al., 2008). As there was no effect of GLPAg on body weight overall but a significant effect was seen on body weight change, this may
suggest that interindividual variability in food intake and body weight led to the lack of significant
difference and that in measures where animals were compared to themselves a significant
difference may be seen.

Chronic treatment with GLPAg in DIO mice appeared to have beneficial effects on glucose tolerance
(Figure 2.17). Baseline glucose was lower in the GLPAg group than in saline controls and GLPAg
caused a reduction in area under the curve seen in a glucose tolerance test. There was no effect of
GLPAg on baseline insulin levels or on the insulin response to a glucose tolerance test. Increased
insulin sensitivity has been previously reported in response to chronic treatment with GLP-1R
agonists and may account for the improved glucose tolerance in the absence of increased insulin
secretion (Mizuno et al., 1997; Young et al., 1999). Relatively small changes in body weight are also
associated with an improvement in insulin sensitivity and could explain the effects of GLPAg on
glucose tolerance (Golay et al., 1985; Bryson et al., 1996; Niskanen et al., 1996). Further work to
investigate the mechanisms of an apparent increase in insulin sensitivity after chronic GLPAg
treatment might include a comparison of insulin sensitivity in an insulin tolerance test with GLPAg
treated and pair fed control animals.

The lack of effect of GLPAg on insulin release in response to a glucose load is perhaps surprising as
GLP-1 is known to enhance glucose-stimulated insulin release in an acute setting (Kreymann et al.,
1987; Mojsov et al., 1987). If GLPAg did increase insulin sensitivity this itself would affect insulin
release (Ahren and Pacini, 2004). The lack of effect of GLPAg on insulin release could also be a result
of the low dose used or of timing of the GTT, which was carried out five hours after the GLPAg
injection. Thus the circulating levels of GLPAg may have fallen below the threshold required to
stimulate insulin release.
2.5.4 Action of GCGAg

GCGAg is a glucagon receptor agonist designed in our laboratory which has an IC50 at the human glucagon receptor similar to that of glucagon itself. It was also found to bind to the human GLP-1R, although with an IC50 approximately two orders of magnitude higher than that of GLP-1.

GCGAg given at a dose of 50 nmol/kg to mice did not reduce food intake over the 24 hour period tested (Figure 2.9). However there was a reduction in food intake in the first hour, and a compensatory increase in food intake in the second hour after injection. This short duration of action could suggest rapid clearance from the circulation. However the pharmacokinetic study showed however that GCGAg remains elevated in the plasma for up to 72 hours after injection. This could suggest that compensatory mechanisms exist which prevent the long term reduction of food intake by the mechanism employed by glucagon receptor agonists. However methodological considerations may also explain this discrepancy (see section 2.5.5.3 for further discussion).

Daily administration of GCGAg to rats over a 7 day period, in conjunction with EX-4, led to reductions in both cumulative and daily food intake over the entire period when compared to saline, suggesting there was no tachyphylaxis over this timescale. There was also a reduction in food intake in the GCGAg + EX-4 group when compared to the EX-4 group. This is perhaps surprising given the lack of efficacy of GCGAg in reducing food intake in mice when administered alone over 24 hours (Figure 2.9). GCGAg was also administered at a high dose (500 nmol/kg) to rats (Appendix 6.2) and no effect on feeding was seen. The apparent discrepancy might be explained if the effects of GCGAg on feeding are enhanced by the combination with EX-4, which would be in keeping with the results of the coadministration of GLP-1 and glucagon study (Figure 2.10) where a combination of both hormones appeared to lead to a greater reduction in food intake than either individually.

The combined administration of GCGAg (100 nmol/kg) and EX-4 (5 nmol/kg) not only caused a reduction in body weight compared to saline controls and EX-4 alone, but also caused a greater reduction in body weight than was seen in the pair-fed control group (Figure 2.14 B). This suggests
that GCGAg induced an increase in energy expenditure, as would be predicted as a glucagon receptor agonist. The effects of GCGAg in the DIO mouse study on energy expenditure are difficult to assess without a pair-fed control group. However as no effect of GCGAg on food intake was seen in this study, and a small but significant change in body weight was seen this may suggest there was some effect on energy expenditure, even at the low (40 nmol/kg) dose used in this study.

Chronic treatment with GCGAg in DIO mice appeared to have beneficial effects on glucose tolerance (Figure 2.17). Baseline glucose was reduced in compared to saline controls the GCGAg treated animals. In addition, GCGAg caused a reduction in area under the curve seen in a glucose tolerance test. This improvement in glucose tolerance is somewhat unexpected, given the reported negative effects of glucagon on glucose tolerance in diabetic patients (Shah et al., 1999; Shah et al., 2000). There was no statistically significant difference in body weight between the saline control and GCGAg treated animals. However a lack of overall change in body weight may mask other changes such as differences in visceral adiposity, which has been shown to have effects on glucose tolerance independent of differences in body weight or subcutaneous adiposity (Pouliot et al., 1992; Borel et al., 2012). Measurements of visceral adipose depots in future studies would be useful to determine whether the peptides have an effect on this important variable.

It may be that the low fasting glucose and reduced area under the curve for blood glucose in GCGAg treated animals is not a reflection on their improved glucose tolerance as such, but rather an artefact of the protocol used in this GTT. Mice were given a dose of their assigned peptide on the morning of the GTT and then were fasted for five hours before the test began (and remained fasted during the test). As GCGAg is a glucagon receptor agonist it may have stimulated glycogenolysis. The fasting period may have limited the ability of these animals to replenish their glycogen stores such that at the time of the glucose injection their glycogen stores were lower than the control and GLPAg groups. Depleted glycogen stores have been shown to increase glucose uptake (Hespel and Richter, 1990; Jensen et al., 1997) and thus the GCGAg treated animals may have had a greater capacity for
glucose uptake, but only because they had greater depletion in glycogen stores. Thus under other experimental conditions, such as an extended fast where all groups are glycogen depleted, may have led to the GCGAg group appearing less different to saline controls. Further discussion of the limitations of glucose tolerance testing by this protocol and in general can be found in section 2.5.5.4.

Although GCGAg is predominantly a glucagon receptor agonist, it does bind to the GLP-1R. It is thus possible that it is this action of GCGAg which prevents a detrimental effect of glucagon receptor activation on blood glucose, and produces the benefit seen. Co-administration of a GLP-1R antagonist with GCGAg, or administration of GCGAg to GLP-1R null mice could be used to investigate this possibility.

2.5.4.1 Effect of combined administration of GLPAg and GCGAg in DIO mice

Combined administration of GLPAg and GCGAg to DIO mice lead to a significant body weight change compared to saline treated controls. Although the change in body weight was not significantly greater than in the groups receiving GLPAg or GCGAg alone, the combination did appear more effective.

Combined administration of GLPAg and GCGAg leads to increased insulin secretion in response to a glucose load compared to saline control animals. This increase in plasma insulin may be responsible for the near absence of alteration to blood glucose in response to a glucose load seen in mice receiving GLPAg + GCGAg. It is possible that the lower baseline insulin levels seen in the GLPAg + GCGAg group when compared to the saline controls and other treatment groups might allow for greater glucose-stimulated insulin release.

2.5.5 Discussion of methodologies used and their limitations

2.5.5.1 Acute feeding studies

As discussed in section 2.5.1, the protocol used in the current studies may not be sensitive enough to detect the anorectic effect of very low doses of peptides with short circulating half-lives. A
protocol measuring first spontaneous meal size rather than interval food intake, or measuring food intake a shorter interval after injection may be more sensitive.

2.5.5.2 Pair-feeding studies

Pair-feeding studies are useful for assessing the contribution of altered food intake to the body weight change seen in treated animals. However pair-feeding studies have inherent limitations. The feeding pattern of peptide treated and pair fed controls will be different which could lead to apparent differences in body weight when weighed at a fixed time of day.

The pair-feeding protocol used in these studies means the pair-fed animals were fed the median weight of food consumed by the peptide treated group. However the food intake in the peptide treated groups showed high interindividual variability, for example on day one of the GCGAg and EX-4 pair-feeding study (Figure 2.14C) the food intake in the GCGAg + EX-4 group ranged from 6.3g to 22.8g. This variation may be due to the difference in starting weights of the animals, where the animal who ate 6.3g weighed only 460g whereas the animal who ate 22.8g weighed 536g. A similar range of body weights exists in the pair-fed group, yet they are all given the same amount of food. Thus the deficit in food available is relatively greater for larger animals.

2.5.5.3 Pharmacokinetic studies

The pharmacokinetic studies shown in this chapter were carried out with the aim of identifying peptides which persist in the plasma for a period of up to 3 days and were part of a programme screening numerous peptides including those discussed in this chapter. The peptides were administered subcutaneously at large doses (1 mg per animal). This relatively high dose of peptide (equivalent to approximately 1000 nmol/kg for a peptide the size of glucagon), is given such that levels will be detectable by RIA in a practical sample size (25 μl plasma).

Like the peptides investigated in this chapter, insulin treatment for diabetics is also administered as a subcutaneous injection which forms a precipitate once injected. The absorption rate of insulin from a subcutaneous depot varies with the concentration at which it was injected such that when
injected at a higher concentration it is absorbed more slowly (Kolendorf et al., 1983; Mosekilde et al., 1989). Since in the pharmacokinetic studies the peptides were injected at a higher concentration than in the feeding studies, it is likely in the feeding studies the peptide is absorbed, and hence cleared, more quickly than the pharmacokinetic studies imply. This may explain the apparent discrepancy between the short duration of action of GCGAg with regards to food intake in the acute feeding study (Figure 2.9), where the pharmacokinetic study implies a longer duration of action (Figure 2.12).

### 2.5.5.4 GTT

GTT protocols vary widely between laboratories and depending on the precise aims of the experiment. One important variation is the method of glucose administration, as this dictates what response is being tested. In the current study I used intraperitoneal administration of glucose. This has the advantage of being relatively simple to perform and appears less stressful for the animal than an oral gavage. Intraperitoneal administration of glucose bypasses the oral cavity and gut, as glucose is absorbed directly into the bloodstream. This means that any effects of the treatment peptide on gastric emptying will not affect the absorption of glucose and the resulting blood glucose level. In addition, anticipatory insulin release, occurring in the preabsorptive stage in response to sweet tasting stimuli (Grill et al., 1984) will not occur after intraperitoneal glucose administration. Some component of the ability of GLP-1 to reduce glucose excursion has been attributed to its effects on gastric emptying (Nauck et al., 1997). These effects will not be picked up in the current study.

The period of fasting before a GTT has been shown to impact on the results of these studies (Andrikopoulos et al., 2008). An overnight or 24 hour fast results in low baseline glucose and insulin values and the suppression of glucose-induced insulin secretion. If baseline glucose values are low in all groups a beneficial effect of a treatment agent may be harder to detect. A fast of around 6 hours
was found to produce the maximum sensitivity in picking up the difference in glucose handling between normal chow fed and high fat diet fed mice.

### 2.5.6 Conclusions and future directions

Glucagon and GLP-1 have short lived anorectic effects when administered to mice. Doses of these hormones, too low to inhibit food intake, are anorectic when administered in combination. The mechanisms of action and effects of these hormones centrally are examined in chapter 3 of this thesis.

The use of glucagon and GLP-1 as anorectic therapeutic agents is not practical due to their short circulating half lives. The GLP-1R agonist GLPAg, and the glucagon receptor agonist GCGAg investigated in these studies can persist in the circulation for at least 48 hours, and the anorectic effect of GLPAg is significant over a 24 hour period. In chronic studies the combination of EX-4 and GCGAg appeared to induce weight loss both by decreasing food intake and increasing energy expenditure. When GCGAg was combined with the GLP-1R agonist GLPAg there also appeared to be a superior weight loss than was seen after administration of the individual agents, and without any significant changes in food intake.

As discussed in the introduction to this chapter, the effect of any GLP-1 and glucagon receptor therapies on blood glucose, both in an acute setting post injection and over a longer period, is crucial to the use of such a therapy to treat obese type 2 diabetics. It appears from the current studies that the GCGAg did not cause deterioration in glucose tolerance, and in fact may cause an improvement. The ratio of GLP-1 to glucagon receptor activation is likely to be of importance in determining the effect of both acute and chronic administration of these peptides on glucose handling due to their opposing effects on blood glucose and is currently being investigated in our laboratory.
3 EXPERIMENTAL CHAPTER TWO

Examination of the receptors and populations of neurons involved in the regulation of feeding by glucagon, GLP-1 and oxyntomodulin.
3.1 INTRODUCTION

3.1.1 The hormonal regulation of appetite

The regulation of appetite by hormones produced in the gut, pancreas and adipose tissue has been well documented (Gardiner et al., 2008; Ahima, 2006; Woods et al., 2006). The study of their mechanisms of action is important to provide a potential explanation of the similarities and differences in effects seen by these evolutionarily related hormones and, increasingly, is required to provide information about the likely effects of synthetic agonists of the GLP-1 and glucagon receptors which are currently being developed with the aim of treating obesity.

To fully understand the anorectic mechanisms of these hormones it is necessary to determine where they act to elicit their effects, and on what receptors. Downstream of binding to the receptor there will be a chain of events leading to a satiety and/or satiation. The location of their receptors and the downstream effects in the CNS vary between hormones, some of which act peripherally, and others at any of numerous central sites. However there are common pathways activated by many, if not all, anorectic hormones and these are discussed in the general introduction (section 1.3.1). In this chapter the mechanisms of anorexia induced by members of the preproglucagon family of peptides will be examined and compared.

3.1.2 Anorectic mechanisms of GLP-1

GLP-1 is released from both the L-cells of the small intestine and a small population of neurons in the brainstem (section 3.1.5.2.3). Whilst GLP-1 from both sources may have a role in regulating feeding, it is not known whether they target a common GLP-1R population. The role of centrally released GLP-1 in the regulation of food intake is discussed in section 3.1.5.2.3. An understanding of the effects of peripherally released or administered GLP-1 is particularly relevant to the development of peripherally administered GLP-1R agonists to be used therapeutically.

The mechanisms by which GLP-1 affects feeding have been the subject of numerous studies and review articles. Intracerebroventricular (ICV) administration of GLP-1 induces c-fos expression in the
hypothalamus (ARC, PVN, SON), brainstem (AP, NTS, PBN) and central nucleus of the amygdala (CeA) (Van Dijk G. et al., 1996; Larsen et al., 1997b). The GLP-1R is expressed in all these regions, and thus centrally administered GLP-1 may be acting on them directly to increase the expression of c-fos (Merchenthaler et al., 1999). Peripheral administration of GLP-1 has been reported to result in the same pattern of activation (Abbott et al., 2005; Asarian, 2009) which might suggest peripherally released GLP-1 crosses the blood brain barrier to have the same effects on neuronal activation as are seen following ICV administration. However not all studies have found activation of hypothalamic nuclei in response to peripherally administered GLP-1 (Baumgartner et al., 2010) and the role of centrally expressed GLP-1Rs in mediating the effects of peripherally released or administered GLP-1 remains contentious (Trapp and Hisadome, 2011; Vrang and Larsen, 2010).

There is evidence that the central effects of peripherally administered GLP-1 may be mediated by GLP-1Rs expressed on vagal afferents, since GLP-1-induced c-fos expression in the ARC is abolished by vagotomy (Abbott et al., 2005). This is consistent with the ability of albumin-conjugated GLP-1, which cannot cross the blood brain barrier, to induce its characteristic c-fos pattern (Baggio et al., 2004a). Peripheral, but not central, administration of EX 9-39 blocks the effects of peripheral GLP-1, suggesting that binding of GLP-1 to central GLP-1R is not necessary to inhibit food intake (Williams et al., 2009).

3.1.3 Anorectic mechanisms of Glucagon

In contrast to GLP-1, the mechanisms by which glucagon affects feeding have not been extensively studied. Of the sites expressing glucagon receptors (see section 1.4.2.3), the liver and CNS appear to be the most likely sites of action.

Glucagon-induced satiety appears to require intact vagal afferents (Geary et al., 1993; Weatherford and Ritter, 1988) yet glucagon receptors have not been identified on these fibres. There is also some evidence for a direct effect of glucagon in the CNS. Glucagon administered ICV inhibits food intake (Inokuchi et al., 1984) and glucagon applied directly to nerve fibres in the of some neurons in the
DMN, VMN and particular glucosensitive neurons in the LHA inhibits their electrical activity in a dose dependent manner (Inokuchi et al., 1986). Inhibition of neuronal firing in the LHA is also observed, although to a lesser extent, when glucagon is administered into the carotid artery or hepatic-portal vein. As the effects of circulating glucagon mimic some of the effects of centrally applied glucagon on the hypothalamus, this implies these effects are likely to be direct rather than mediated by vagal activation.

It has been suggested that glucagon might act indirectly to inhibit food intake by inducing hyperglycaemia (Martin and Novin, 1977; Vanderweele et al., 1979). This hypothesis is countered by a study showing that a dose of glucagon capable of causing hyperglycemia does not reduce food intake during sham feeding in rats (Geary and Smith, 1982a).

Glucagon stimulates insulin release (Kawai et al., 1995), and insulin is known to inhibit appetite (Woods et al., 1979). To determine whether glucagon-induced insulin release is responsible for glucagon mediated anorexia, glucagon was co-infused with insulin antibodies (Geary et al., 1997). Rats given an infusion of glucagon and the insulin antibody ate less than animals given the insulin antibody alone, thus neutralisation of insulin did not prevent the anorectic effects of glucagon.

There is no information available about the receptor at which glucagon acts to exert its anorectic effect. To date food intake after glucagon administration has not been measured in a glucagon receptor knockout mouse or in the presence of a glucagon receptor antagonist. The evolutionary relationship and structural similarity between glucagon and GLP-1 might suggest that glucagon acts via the GLP-1R to reduce feeding, and indeed, there is some cross reactivity between glucagon and the GLP-1R. The IC$_{50}$ for glucagon binding at the human GLP-1R is between 1 and 10 μM compared to the IC$_{50}$ of GLP-1 which is between 0.3 and 1 nM (Druce et al., 2009; Thorens, 1992). Given this large difference in affinity for the GLP-1R, the relatively small difference in potency between GLP-1 and glucagon for food intake inhibition would suggest that glucagon cannot be acting solely at the GLP-1R to inhibit feeding. In addition to the affinity of a peptide for a receptor, the pharmacokinetics of a
peptide affects its \textit{in vivo} potency. Glucagon has been reported to have a longer half life than GLP-1 in rats (Oshima et al., 1988) and this should be taken into account when considering the relative doses required for GLP-1 and glucagon to inhibit feeding.

3.1.4 Anorectic mechanisms of Oxyntomodulin

As an agonist at both the GLP-1 and glucagon receptors, oxyntomodulin might be expected to affect feeding by the same mechanisms as these two hormones, although the lack of mechanistic information about glucagon-induced anorexia makes this hard to assess.

The effect of oxyntomodulin on neuronal activation in appetite regulating centres has been examined by measurement of c-fos- like immunoreactivity. c-fos expression was seen following peripheral administration of oxyntomodulin in the PVN and ARC in the hypothalamus and NTS and AP in the brainstem (Baggio et al., 2004b; Dakin et al., 2004).

The anorectic effects of oxyntomodulin appear to be mediated by activation of the GLP-1R (Baggio et al., 2004b). Comparison of the neuronal activation in response to anorectic doses of GLP-1 and oxyntomodulin is one means of determining whether their activation of a common receptor is reflected in a wholly shared mechanism of action. There are no published reports of a direct comparison of the c-fos expression induced by GLP-1 and oxyntomodulin. Baggio et al. carried out a comparison of c-fos expression in response to oxyntomodulin and the GLP-1R agonist EX-4. Baggio et al. found peripherally administered EX-4 and oxyntomodulin to activate the same brain regions (PVN, AP and NTS) (Baggio et al., 2004b). As the doses of EX-4 and oxyntomodulin used in this study were not equivalently anorectic, and EX-4 does not appear to bind to the GLP-1R in the same way as GLP-1 itself (Barrera et al., 2009). Further work is needed to determine whether the c-fos expression induced by GLP-1 and oxyntomodulin is identical. This question is addressed in experiments 3.3.3 and 3.3.7.
3.1.5 The brainstem and central nucleus of the amygdala

The work carried out in this chapter focused on the brainstem and in particular, on the NTS. A discussion of the anatomy and physiology of this region with relation to food intake is given below. In addition the peptides examined in this chapter also induce c-fos expression in the CeA, which has numerous bidirectional connections to the brainstem. This region is also discussed below.

3.1.5.1 The Nucleus Tractus Solitarius

The solitary tract is a bundle of fibres composed of sensory axons of the VIIth, IXth and Xth cranial nerves. Surrounding this fibre bundle is the NTS. The NTS is composed of numerous subnuclei and extends rostrally from its caudal portion (bregma -8.24 mm) to (bregma -6.24 mm) in the mouse brainstem (Figure 3.1). Sensory afferents from diverse peripheral systems including the heart, lungs, gastrointestinal system, skeletal muscle and reproductive organs are relayed in the NTS (Gwyn et al., 1985; Leslie et al., 1982; Shapiro and Miselis, 1985a; Donoghue et al., 1984; Davies et al., 1987; Hubscher and Berkley, 1994).

Projections from the periphery to the NTS are thought to terminate in both ‘organ-specific’ subnuclei and also to project to areas of the NTS receiving inputs from multiple peripheral sources (Loewy A.D., 1990). Subnuclei receiving ‘organ-specific’ projections are thought to be involved in reflex control of organ function. One such reflex begins with stretch receptor afferents projecting to the ventral and ventrolateral subnuclei in the intermediate portion of NTS. Here they make monosynaptic connections with a population of neurons which project to motor neurons in the phrenic nucleus of the spinal cord controlling the movement of the diaphragm (Backman et al., 1984; Lipski et al., 1983).

The caudal (also called commissural) NTS, contains sensory axons terminals from cardiovascular, respiratory and gastrointestinal sensory afferents (Kalia and Mesulam, 1980). The convergence of inputs from multiple peripheral sources at this NTS subnucleus is hypothesised to be involved in mediating more complex integrative responses such as behavioural changes (Loewy A.D., 1990), and,
consistent with this, the commissural NTS has ascending projections to forebrain areas (Ter Horst et al., 1989).

![Diagram showing subdivisions of the NTS and their projections](image)

**Figure 3.1: Schematic showing subdivisions of the NTS and the names by which they are described in this thesis.** These regions are defined as in (Barraco et al., 1992). Nucleus Tractus Solitarius (NTS), Area postrema (AP), 4th ventricle (4V).

From the NTS projections ascend from both the rostral and more caudal portions to the PBN, with some fibres branching off to terminate at ventrolateral medullary targets before reaching the PBN. Most projections from the rostral portion of the NTS terminate in the PBN, along with some axons originating in the more caudal portions. The majority of projections from the more caudal portions ascend further, splitting into two paths in the region of the periaqueductal grey. Most of these projections ascend within the medial forebrain bundle (MFB); the remainder are carried in the periventricular grey. From the MFB projections terminate in the DMH, PVN, ARC, SON, and LHA in the hypothalamus, amygdala, and bed nucleus of the stria terminalis (BNST). Projections ascending via the periventricular grey terminate mostly in the hypothalamic periventricular nucleus, with a few terminating in the VMN (Ter Horst et al., 1989).
The NTS also receives many descending projections. Projections from the prefrontal cortex are found to terminate throughout the rostro-caudal extent of the NTS, and are largely in the dorsal portion. Subcortical projections from the BNST, CeA and hypothalamus (PVN and ARC) were also found to terminate throughout the rostro-caudal extent of the NTS, but were mostly found ventrally and laterally (van der Kooy D. et al., 1984).

As well as projecting extensively to other parts of the brain, neurons from NTS subnuclei are interconnected such that it is likely that the different subnuclei are able to influence one another’s activity (Norgren, 1978).

3.1.5.1.1 Gustatory processing in the NTS

The caudal brainstem is important for taste processing as shown by the ability of decerebrate animals to discriminate between palatable and unpalatable flavours (Grill, 1980). Taste afferents ascend via the facial, glossopharyngeal and vagus nerves from specific regions of the mouth, tongue and nasal cavity, and are mapped onto corresponding zones in the rostral NTS (Travers et al., 1986). From the rostral NTS there are dense projections to the pontine taste area in the medial PBN, from which projections carry gustatory information to the thalamus and subsequently to the cerebral cortex (Loewy A.D., 1990). The response of the NTS to a sweet taste stimulus, as assessed by measurement of multiunit electrical activity, is reduced after intrahepaticportal administration of glucagon (Giza et al., 1993) suggesting the rostral part of the NTS may be activated by peripherally administered glucagon.

3.1.5.1.2 Gastrointestinal sensory processing in the NTS

Sensory afferents from the GI tract project via the vagus nerve to the subpostremal (also called the parvocellular or gelatinosa) and caudal NTS, and afferents projecting from different parts of the GI tract appear to terminate in discrete regions (Rogers and Hermann, 1983; Shapiro and Miselis, 1985a; Norgren and Smith, 1988; Altschuler et al., 1989). Some of these sensory afferents carry information.
about gastric and intestinal distension (Zhang et al., 1992; Zhang et al., 1995). Gut peptides such as CCK are also thought to stimulate sensory afferents terminating in the NTS (Appleyard et al., 2005).

3.1.5.1.3 Glucose sensing in the NTS

Glucose sensitive neurons have been identified in the caudal portion of the NTS, many of which appear to be catecholaminergic (Adachi et al., 1984; Mizuno and Oomura, 1984; Yettefti et al., 1997). A fuller description of the activity of these neurons and their physiological role can be found in section 3.1.5.2.1.

3.1.5.2 NTS neuronal populations

Within the NTS a variety of neurotransmitters and neuropeptides are expressed (Barraco et al., 1992). Work in this thesis focused on four distinct populations within the caudal part of the NTS: POMC, GLP-1, Tyrosine Hydroxylase (TH), and CART expressing neurons (Figure 3.2).

Figure 3.2: Four immunohistochemically distinct neuronal populations in the caudal NTS involved in appetite regulation. Taken from Anatomy and regulation of the central melanocortin system (Cone, 2005). AP area postrema, NTS, nucleus tractus solitarius, Sol solitary tract, SoIC commissural nucleus of the solitary tract, CC central canal, DMV Dorsal motor nucleus of the vagus, SolM medial nucleus of the solitary tract, POMC proopiomelanocortin, TH tyrosine hydroxylase, GLP-1 Glucagon-like peptide-1, CART cocaine-and amphetamine-regulated transcript protein.
3.1.5.2.1 Catecholaminergic (TH expressing) neurons

Catecholaminergic (CA) neurons are found in multiple locations in the NTS. The C2 adrenaline cell group is found mostly in rostral NTS, but is also found in the subpostremal portion of this nucleus. The A2 noradrenaline cell group is found from the region just rostral to the obex extending caudally into the spinal cord (Barraco et al., 1992). Several studies have found CA neurons in the NTS to be activated by stimuli related to feeding. Vagal afferents have monosynaptic connections to CA neurons in the NTS and the firing of CA neurons was facilitated by CCK application in vitro (Appleyard et al., 2007). Distension of the proximal colon also activates some CA neurons in the NTS (Wang et al., 2009). Central and peripheral administration of the GLP-1R agonist EX-4 has been shown to induce c-fos expression in a portion of CA NTS neurons (Yamamoto et al., 2002). In contrast the orexigenic hormone ghrelin inhibits the firing of NTS CA neurons in vitro (Cui et al., 2011). Noradrenergic projections are found from the A2 cell group to the PVN and CeA (Sawchenko and Swanson, 1981; Reyes and Van Bockstaele, 2006). The response of these neurons to gut hormones, and to a GLP-1R agonist in particular, make them promising candidates for activation by the PPG-derived peptides.

3.1.5.2.2 POMC expressing neurons

POMC expressing neurons are found in the subpostremal and caudal parts of the mouse NTS (Joseph et al., 1983; Bronstein et al., 1992; Appleyard et al., 2005). α-MSH, ACTH and β-endorphin immunoreactivity have all been detected in these cells (Palkovits et al., 1987). NTS POMC neurons project to the lateral PBN, but the majority of axons terminate within the NTS itself (Joseph et al., 1983).

NTS POMC neurons have also been implicated in the regulation of feeding. Feeding-induced satiety causes c-fos to be expressed in these neurons (Fan et al., 2004) and induction of c-fos immunoreactivity is seen in around 30% of NTS POMC neurons after IP CCK administration (Appleyard et al., 2005). The NTS POMC neurons are also responsive to peripherally administered
leptin (Ellacott et al., 2006). The location of these neurons in the subpostremal region of the NTS and their response to circulating regulators of satiety makes them candidate downstream targets of the PPG derived peptides.

### 3.1.5.2.3 Preproglucagon expressing neurons

Preproglucagon (PPG) is expressed in a population of neurons in the caudal and subpostremal NTS (Figure 3.3). Cell bodies are also found in the intermediate reticular nucleus, at the ventral border of the hypoglossal nucleus and a very few neurons in the olfactory bulb (Larsen et al., 1997a; Merchenthaler et al., 1999; Llewellyn-Smith et al., 2011). Prohormone convertases (PC) 1/3 and 2 are expressed in the NTS (Schafer et al., 1993) but no investigations of potential colocalisation of either enzyme with PPG gene products have been published. It appears however, from studies looking at which PPG gene products are produced in the NTS, that PC 1/3 processing predominates in these neurons. GLP-1, GLP-2, oxyntomodulin and glicentin predominate, with only small amounts of glucagon being produced (Larsen et al., 1997a; Lovshin et al., 2004).

At the current time no full characterisation of the neuropeptides expressed by PPG neurons has been carried out. However it has been shown that they are distinct from neurons expressing catecholamines (Jelsing et al., 2009) and those expressing POMC (Fan et al., 2004). There has been some suggestion that they may also express somatostatin although the data is yet to be published (Vrang and Larsen, 2010).
Figure 3.3: Preproglucagon neurons in the mouse brainstem. Immunohistochemistry for yellow fluorescent protein (YFP) expressed in this mouse under the PPG promoter (Llewellyn-Smith et al., 2011). Area Postrema: AP, nucleus tractus solitarius: NTS, central canal: cc, hypoglossal nucleus: HGN, intermediate reticular nucleus: IRT. A: YFP-immunoreactive cell bodies in the cNTS, IRT and the ventral border of the HGN, (arrow) and in the raphé obscurus (arrow) Scale Bar=250 um. B: The cNTS containing YFP-immunoreactive cell bodies and a dense network of dendrites. Scale Bar, 100 um. C: The IRT. Scale Bar, 100 um.

3.1.5.2.3.1 Projections

The NTS PPG neurons project extensively to the PVN where there appear to be numerous appositions between PPG neurons and cell bodies expressing oxytocin or CRH (Jin et al., 1988; Larsen et al., 1997a; Rinaman, 1999b; Tauchi et al., 2008). Elsewhere in the hypothalamus GLP-1 immunoreactive projections are seen in the DMH, SON and medial preoptic area (Jin et al., 1988; Vrang et al., 2007). A recently developed transgenic mouse expressing yellow fluorescent protein (YFP) under the PPG promoter has allowed a more comprehensive mapping of the projections of
PPG neurons to be carried out (Llewellyn-Smith et al., 2011). Using this mouse, dendritic and axonic projections to the AP were visualised. Axons in the NTS were most dense caudally with a few axons in the intermediate NTS. Projections of PPG neurons were seen in much of the ascending visceral pathway from the NTS extending within the brainstem to the rostral ventrolateral medulla, periaqueductal grey and locus coeruleus. In the hypothalamus dense projections were found in the PVN and DMN, with moderate numbers in the LH and caudal ARC, and a few axons in the VMH. Forebrain PPG projections were seen in the thalamus, organism vasculosum of the lamina terminalis and a few projections to the amygdala.

3.1.5.2.3.2 Physiological roles

Evidence suggests the PPG neurons are involved in feeding. c-fos expression in these neurons has been documented in response to food intake, gastric distension and CCK administration (Rinaman, 1999b; Vrang et al., 2003; Gaykema et al., 2009). Post natal knockdown of PPG gene expression in the NTS using RNA interference in rats caused them to become hyperphagic and heavier than wild type controls, suggesting the release of PPG gene products from the NTS is physiologically relevant to food intake (Barrera et al., 2011).

PPG neurons are also activated by peripheral administration of leptin, express leptin receptors, increase PPG expression in response to central leptin administration and depolarise in response to leptin in vitro (Goldstone et al., 2000; Elias et al., 2000; Goldstone et al., 1997; Hisadome et al., 2010). The GLP-1R antagonist EX 9-39 effectively reduced the response to centrally administered leptin, suggesting centrally released GLP-1 is an important mediators of leptin’s central effects on feeding (Goldstone et al., 1997). The ability of PPG neurons to respond to leptin as well as signals of food intake suggests that the PPG neurons might alter their response to short term satiety signals dependant on the body’s fat stores and therefore may represent a point of integration between long and short term signals of nutrient availability.
In addition to meal and adiposity related signals, PPG neurons appear to have a role in the stress response. c-fos expression is induced in PPG neurons by peripheral administration of lipopolysaccharide or lithium chloride in rats and mice (Rinaman, 1999b; Lachey et al., 2005). Peripherally administered LiCl inhibits feeding and induces conditioned taste aversion, but both responses were blocked by a centrally administered GLP-1R antagonist in rats (Rinaman, 1999a; Seeley et al., 2000) suggesting that PPG neurons are important in mediating both the anorexia and the aversion produced as a result of interoceptive stressors. However GLP-1R knockout mice respond normally to LiCl, which the authors of the study suggest shows a species difference in the role of central GLP-1Rs in mediating LiCl induced nausea between mice and rats (Lachey et al., 2005). Thus PPG neurons may also be involved in LiCl induced anorexia and aversion in mice, but receptors other than the GLP-1R are likely to be sufficient for the response.

### 3.1.5.3 The parabrachial nucleus

The PBN receives extensive projections from the NTS (Herbert et al., 1990) and the spatial separation of different types of sensory afferents seen in the NTS is somewhat maintained at the level of the PBN. The PBN is thought to be involved in gustatory processing. First order taste neurons from the NTS project to the PBN and from there second order taste neurons project to the hypothalamus and amygdala amongst other areas (Norgren, 1976; Norgren and Leonard, 1973). The PBN also has bidirectional connections to reward centres (Lundy and Norgren R, 2004) and is thought to be important for the response to palatable foods.

### 3.1.5.4 The central nucleus of the amygdala

There is a clear association between stimuli which affect feeding and the amygdala, with increased amygdaloid c-fos expression seen in response to a range of anorectic stimuli (Olson et al., 1993). The CeA is involved in associative learning and related to this, in fear and anxiety (Davis, 1992; Petrovich and Gallagher, 2003). Projections from the CeA to the LHA and DMV are thought to be involved in the activation of the sympathetic nervous system during anxiety (Davis, 1992) whereas projections
to the PVN and SON are thought to be involved in the neuroendocrine response (Sawchenko and Swanson, 1983). The amygdala is thought to be key to the effects of stress on memory (McGaugh and Roozendaal, 2002). The role of the amygdala in feeding-associated learning is somewhat complicated. CeA lesions do not affect food consumption in response to learnt appetitive cues, whereas lesions of the basolateral amygdala prevent this response (Petrovich and Gallagher, 2003). In contrast CeA lesions abolish the ability of a negative stimulus such, as a shock, to cause conditioned aversion to feeding (Petrovich et al., 2009). Control of feeding by aversive cues is unaffected by lesions of the basolateral amygdala. The CeA also projects to dopaminergic neurons in the VTA, connecting it to important reward centres in the brain (Beckstead et al., 1979).

The majority of projections from the CeA are thought to be GABAergic (Swanson and Petrovich, 1998). Amongst the neuropeptides expressed by neurons in the CeA are a population expressing CRH (Asan et al., 2005). These have been linked to food intake in a few studies. The levels of CRH measured in samples collected from the extracellular fluid in the CeA of rats by microdialysis sampling increased after feeding suggesting release of CRH either from cells in the CeA or projections to this nucleus (Merali et al., 1998). The levels of CRH mRNA fall in the CeA after 12 hour food deprivation in rats and are raised after refeeding (Timofeeva and Richard, 1997; Timofeeva et al., 2002).

3.1.6 Background on experimental techniques and models used in this chapter

3.1.6.1 Immunohistochemistry

Immunohistochemical identification and characterisation of activated neurons is one method of investigating the effects of peripherally administered peptides on the CNS and can help to elucidate their mechanisms of action. Immunohistochemistry (IHC) uses an antibody raised against the epitope of interest which is applied to the tissue and then, using an appropriate method of detection, used to localise that epitope. A major advantage of this technique over other methods of determining neuronal activation, such as functional magnetic resonance imaging (fMRI), is that
rather than merely identifying broad areas of activation, the resolution is such that individual activated neurons can be seen, and if desired, characterised by staining for markers specific to a chosen neuronal subtype. The sensitivity and specificity of staining by IHC is determined by a number of factors, some of which are discussed below.

3.1.6.1.1 Antibodies

Antibodies for IHC are raised by injection of a purified or synthetic antigen, often with an adjuvant, into a host species. The resultant antiserum contains a heterogeneous mixture of antibodies against several different epitopes of the administered antigen, known as polyclonal antibodies. The disadvantages of this method of antibody production include the difficulty of reproducing the same antiserum once the limited amount which can be obtained from one host has been exhausted. In addition there will also be other antibodies present in the serum (although these can be removed using affinity purification). The alternative is to use monoclonal antibodies (Kohler and Milstein, 1975). Monoclonal antibodies are produced by fusion of a B-lymphocyte taken from an immunised animal with a non-secreting myloma cell, leading to the production of an immortalised cell line which produces only the antibody produced by the original B-lymphocyte. As each B-lymphocyte only produces one type of antibody, the antibodies produced using this method all bind to a single epitope. Unfortunately monoclonal antibodies have their own disadvantages, chiefly the reduction in sensitivity associated with only binding to a single epitope which, in cases where fixation may alter or mask certain epitopes, can result in no staining.

3.1.6.1.2 Preparation of tissues for IHC

In order for a tissue to be used for IHC it must be fixed, to immobilise the antigen and preserve tissue architecture and cellular morphology, then sectioned to allow for adequate antibody penetration into the tissue. Formaldehyde is the most commonly used fixative. It reacts with various sites on biological molecules causing cross-linking between adjacent molecules (Fox et al., 1985).
This cross linking can alter the ability of antigens to be recognised by antibodies which were raised against the free antigen. Pre-treatment of sections of tissue, known as antigen retrieval, can be used to restore antigens to a state in which they can be recognised by their antibodies if antigen masking by formaldehyde fixation is thought to be a problem.

Various methods of antigen retrieval have been suggested, and the method used may be specific to the antigen to be detected. Enzymatic antigen retrieval to break bonds formed between molecules is usually not favoured due to the possibility of cleavage of prohormones or other molecules to reveal sites which are not usually present in the tissue leading to false positives. Heat mediated antigen retrieval is more common, where the sections are heated in an acidic or alkaline buffer solution (D'Amico et al., 2009).

3.1.6.1.3 Detection of the antibody-antigen reaction

To visualise the site of antibody-antigen reaction either enzymes catalysing chromogenic reactions, or fluorophores are used. Enzymatic detection methods have the advantage of not being susceptible to bleaching in the same way as fluorophores and being detectable without the need for specialised equipment.

Primary antibodies are not usually conjugated directly to the enzyme used for detection as this method is not very sensitive. Instead amplification of the signal can be achieved using a three step avidin-biotin complex (ABC) method (Bayer et al., 1976; Guesdon et al., 1979). Avidin is a large glycoprotein with four high affinity binding sites for biotin, whilst each biotin molecule has only one binding site for avidin. The ABC method uses horseradish peroxidise (HRP) conjugated biotin, which can bind to any of four sites on the large glycoprotein avidin forming a large complex. A secondary antibody conjugated to biotin is used to link the complex containing multiple HRP enzymes to the site of primary antibody-antigen reaction. Thus each primary antibody becomes associated with a larger number of enzymes amplifying the signal.
3.1.6.2 c-fos

The c-fos protein is encoded by the proto-oncogene c-fos. The protein was first identified in the retrovirus Finkel-Biskis-Jinkins murine osteogenic sarcoma virus (FBJ-MSV) where it is known as v-fos, denoting its viral origin (Curran et al., 1983). However, as is common for retroviral oncogenes, v-fos was derived originally from the cellular genome where it is known as the proto-oncogene c-fos.

In most cell types levels of c-fos mRNA and protein are relatively low. Induction of c-fos protein expression can occur due to the presence of growth factors (Greenberg et al., 1985; Kruijer et al., 1985) and was thus thought originally to be involved in regulation of the cell cycle, although it was later found to be associated with cell differentiation in the absence of cell division in response to growth factors (Curran and Morgan, 1985). The induction of c-fos was found to precede the activation of c-myc, making c-fos expression the earliest known element of the pathway in growth factor signalling leading to gene transcription (Muller et al., 1984).

Transcription of the c-fos gene starts within 5 minutes of stimulation and continues for 15-20 minutes, with mRNA levels peaking at 30 minutes (Greenberg and Ziff, 1984). The mRNA concentration is dependent on the dose of the stimulating factor (Greenberg et al., 1985). The levels of c-fos protein appear to peak around 1-2 hours after stimulation and decline rapidly thereafter (Muller et al., 1984). Protein synthesis inhibitors appear to have no effect on c-fos transcription (Greenberg et al., 1985), thus the machinery required for its transcription is present in the resting state. The ability of c-fos to be transcribed in response to an extracellular signal without the need for de novo protein synthesis, lead to its designation as an immediate early gene.

3.1.6.2.1 The c-fos gene

The c-fos gene is located on chromosome 14 in humans and chromosome 12 in mice and contains four highly conserved exons. Some key regulatory features of the c-fos gene are shown in Figure 3.4. Transcription of the c-fos gene is regulated by the binding of transcription factors such as STATs and TFII-I which both bind to the c-Sis inducible element (SIE), serum response factor (SRF) which binds
to the serum response element (SRE), and CREB which binds to the calcium response element (CRE). The regulation of c-fos transcription by CREB ties c-fos expression to intracellular Ca\(^{2+}\) levels which may be altered as the result of cell surface receptors activating a second messenger system or due to an alteration in cell permeability to Ca\(^{2+}\) as may occur during a cellular depolarisation.

**Figure 3.4: Key features of the c-fos gene.** SIE c-Sis inducible element, TCF ternary complex factor, SRE serum response element, AP1/CRE activating protein 1/calcium response element, CRE calcium response element, E1-E4 exons 1-4, ARE AU rich element.

The AU rich element (ARE) in the 3’ untranslated region of the c-fos gene, which targets the mRNA for degradation, is thought to be responsible for the short half life of c-fos mRNA and is present in the 3’ untranslated region of many immediate early genes (Chen and Shyu, 1994; Lee et al., 1988).

**3.1.6.2.2 The c-fos protein**

c-fos is a 380 amino acid protein which is found in the cell nucleus (Curran et al., 1984), can bind to DNA (Sambucetti and Curran, 1986) and activate gene expression (Setoyama et al., 1986). c-fos forms a dimer with another immediate early gene, c-jun forming the transcription factor AP-1 (Curran and Morgan, 1985) and by binding to AP1 sequences in the promoter regions of numerous genes, regulate their transcription.

**3.1.6.2.3 Downstream effects of c-fos expression**
The transcription of many genes expressed by neurons, including those which code for neurotransmitters and neuropeptides, are regulated by c-fos (Wu et al., 2004b; Dong et al., 2006; Hunter et al., 1995; Sabban et al., 1995; Kovacs and Sawchenko, 1996). c-Fos may stimulate or inhibit gene transcription depending on whether if forms a complex with c-Jun or JunB respectively (Chiu et al., 1989; Schutte et al., 1989). c-fos may also play a role in response to ischaemia and
excitotoxic insults (Walker and Carlock, 1993) and there is an association between c-fos expression and neuronal cell death.

### 3.1.6.2.4 c-fos and neuronal activation

c-fos expression can be induced by neuronal activation, such as that caused by stimulation of acetylcholine receptors (AChR) (Greenberg et al., 1986) and particularly with the activation of NMDA receptors (Bading et al., 1993). Stimulation of c-fos expression by nicotinic AChR agonists is dependent on an influx of extracellular calcium. In addition many neuropeptides have been shown to induce patterns of c-fos expression in the brain including corticotrophin releasing hormone (Imaki et al., 1995), angiotensin II (Rowland et al., 1996), GLP-1 (Yamamoto et al., 2002), CART (Vrang et al., 1999), and AgRP (Hagan et al., 2001).

In addition to activation of neurons by binding of classical neurotransmitter or neuropeptide ligands to their receptors on the post synaptic membrane, c-fos expression can be influenced by other signalling modalities. Growth factor stimulated c-fos induction does not require an increase in intracellular calcium, but rather acts via intracellular signalling cascades to modify the binding of transcription factors to c-fos regulatory sequences (Greenberg et al., 1986; Segal and Greenberg, 1996). The ligand-bound glucocorticoid receptor binds directly to the SRE site on the c-fos promoter reducing transcription (Karagianni and Tsawdaroglou, 1994). It is thought that the glucocorticoid receptor acts at this site to block the binding of positive transcription factors and thus may affect the c-fos expression seen in response to other stimuli.

Interactions between multiple stimuli, applied at the same time or successively, have been shown to affect the c-fos expression induced. A synergistic effect on c-fos expression in some thalamic nuclei was seen when rats received a noxious stimulus 90 minutes after receiving an identical noxious stimulus (Redburn and Leah, 1997). In other nuclei examined the presence of a prior noxious stimulus eliminated the c-fos expression seen after a second noxious stimulus. Concomitant activation of D1 and D2 receptors in the striatum appears to have a synergistic effect on c-fos
expression (LaHoste et al., 1993). The differential effects of multiple stimuli on c-fos expression suggest that the integration of these stimuli occurs upstream of c-fos activation and that c-fos expression might be used to assess and compare the effects of single and combined stimuli on the response of neurons.

3.1.6.2.5 The suitability and limitations associated with the use of c-fos as a marker of neuronal activation

If c-fos is to be used as a marker of neuronal activation it is important to consider what elevated c-fos expression means in terms of what has triggered it and what the expected response to c-fos within that neuron would be. As discussed above c-fos expression can be induced in response to a wide range of stimuli, and can be differentially induced in response to multiple stimuli suggesting c-fos would be a suitable marker for neuronal activation in response to single and combined stimuli.

Not all stimuli are associated with an increase, or alteration of any kind, to c-fos expression. High resting activity such as that of the visual or auditory cortices is not associated with high baseline c-fos expression (Kaczmarek and Chaudhuri, 1997; Campeau and Watson, 1997). Although this is perhaps an advantage when mapping changes in neuronal activity in response to external stimuli, it does raise questions about the relevance of c-fos activation in the context of physiologically relevant stimuli. Conversely there are other areas with high constitutive c-fos expression such as the suprachiasmatic nucleus in which changes in expression in response to a stimulus will be more difficult to detect (Chan et al., 1993). Many neurons are under tonic inhibition under resting conditions, and disinhibition of their firing in response to a stimulus produces c-fos expression in some (Cole and Sawchenko, 2002; Zaretskaia et al., 2008) but not all cases (Hoffman et al., 1993). Furthermore there are some examples of neuronal populations or stimulus types which produce no increase in c-fos expression, even though the neurons are activated (Herdegen et al., 1992; Jung et al., 1998; Wisden et al., 1990). In many of these cases other IEGs are expressed in response to the stimuli and are responsible for stimulus-transcription coupling.
The lack of c-fos expression in response to neuronal activation in some cases is not unexpected. Although c-fos is involved in the regulation of expression of numerous genes in neurons and is often upregulated in response to depolarisation, it is not required for neuronal transmission. It is thought that the c-fos expression induced by depolarisation of a neuron is due to elevated intracellular Ca\(^{2+}\). If intracellular Ca\(^{2+}\) does not rise in response to a particular stimulus or in a particular neuronal population, c-fos expression may not be elevated, despite the fact that the neuron has been ‘activated’.

Despite the caveats mentioned above measurement of c-fos immunoreactivity remains a widely used method for assessing neuronal activation. The spatial resolution achievable using c-fos immunohistochemistry is far superior to functional MRI (fMRI) or even the more spatially accurate MEMRI imaging modalities. The ability not only to see individual activated neurons, but to use dual immunohistochemistry to identify other proteins expressed within those neurons, enables more detailed studies of the response of the brain to external stimuli than can be currently carried out using MRI imaging. Thus, although it is not a universal marker for neuronal activation, the measurement of c-fos expression remains an important method for examining the effects of a stimulus on the CNS.

### 3.1.6.3 Models of an absence of GLP-1 receptor signalling

#### 3.1.6.3.1 Exendin 9-39: a GLP-1 receptor antagonist

Exendin 9-39 (EX 9-39) is a truncated form of EX-4 and an antagonist of the GLP-1R, which has an affinity for the GLP-1R similar to that of GLP-1 itself (Goke et al., 1993). EX 9-39 reduces the insulin response to orally ingested glucose in rats (Kolligs et al., 1995; Edwards et al., 1999) and can also affect food intake and body weight (Meeran et al., 1999). It appears that as well as acting as a competitive antagonist at the GLP-1R in the presence of an agonist, EX 9-39 also acts as an inverse agonist: inhibiting the constitutive activity of this receptor, although this has only been shown for the murine receptor to date (Serre et al., 1998).
The family B GPCRs are structurally similar and as a result it is possible that EX 9-39 might cross react with other receptors in this family. This has been demonstrated at the GIP receptor, where EX 9-39 has been shown to bind with micromolar affinity and to antagonise the action of GIP (Gremlich et al., 1995; Wheeler et al., 1995; Gault et al., 2003). Potential cross-reactivity with other receptors is a pertinent drawback of the use of EX 9-39 to study the physiological role of the GLP-1R.

### 3.1.6.3.2 The GLP-1 receptor knockout mouse

The GLP-1R knockout mouse was created in Dr Daniel Drucker’s laboratory at the University of Toronto (Scrocchi et al., 1996). Exons encoding the first and third transmembrane domains of the GLP-1R were replaced with a construct containing the gene for neomycin phosphotransferase under a universal promoter (phosphoglycerate kinase promoter). The original line was created from using the R1 embryonic stem cell line on a CD1 background.

The phenotype of the GLP-1R knockout mouse has been extensively studied. GLP-1R knockout mice are not significantly heavier than CD1 mice at any point measured up to six months of age (Scrocchi et al., 1996) or between eleven and sixteen months of age, although the GLP-1R knockout females appeared slightly, but not significantly heavier (Scrocchi and Drucker, 1998). On 18 weeks of high fat diet, female, but not male, GLP-1R knockout mice had a reduced percentage body weight gain compared to CD1 mice, although their final body weights at the end of 18 weeks of high fat feeding were not significantly different (Scrocchi and Drucker, 1998). Food intake after a 20 hour fast appeared not to be significantly different between CD1 mice and GLP-1R knockout mice, although there was perhaps a small, non significant, reduction in food intake in the knockout animals (Scrocchi et al., 1996). GLP-1 does not reduce food intake in GLP-1R knockout mice.

GLP-1R knockout mice have mildly impaired glucose tolerance compared to wild type CD1 mice, which is more marked in females than in males (Scrocchi et al., 1996; Scrocchi and Drucker, 1998). There is some evidence of haploinsufficiency in heterozygous GLP-1R knockout mice, which exhibit slight impairment to glucose tolerance in oral glucose tolerance tests (Scrocchi et al., 1998). The
impairment of glucose tolerance is seen in GLP-1R knockout mice in both oral and intraperitoneal glucose tolerance tests, where GLP-1R knockout mice show lower plasma insulin levels than CD1 controls (Preitner et al., 2004). There appears to be no reduction in pancreatic insulin mRNA levels or alteration to glucose utilisation in GLP-1R knockout mice (Scrocchi et al., 1998), suggesting the impaired glucose tolerance of these mice is likely due exclusively to the loss of the incretin effect of GLP-1. In support of this it has been shown that GLP-1R knockout mice have normal pancreatic insulin content and respond to glucose by releasing insulin in a manner indistinguishable from wild type islets (Flamez et al., 1998; Preitner et al., 2004). However while isolated islets from GLP-1R knockout mice respond normally to glucose, whole perfused pancreata display reduced first and second phase insulin release from in response to glucose, suggesting an underlying defect (Preitner et al., 2004). The authors of this study propose that, as in their perfused pancreas model the anatomical connections of the pancreas are intact, GLP-1Rs present in a hepatoporal glucose sensor can affect insulin release, and that the loss of these receptors in the GLP-1R knockout is responsible for the impaired insulin response to administered glucose.

Despite most of the evidence suggesting the function of the islets themselves is normal, there have been shown to be some differences in the islets of GLP-1R knockout mice, which despite having normal total beta cell volume and number, have been shown to have a lesser proportion of large islets, and the alpha cells, which are distributed around the islet periphery in wild type mice, were found more often in the centre of islets in GLP-1R knockouts (Ling et al., 2001). The functional implications of this, if any, are unknown. The islets of GLP-1R knockout mice do appear to be more sensitive to destruction by streptozotocin than those of wild type animals (Li et al., 2003).

Compensatory changes in other glucoregulatory hormones are thought to occur in the GLP-1R mouse. Serum gastric inhibitory polypeptide (GIP) levels are increased in GLP-1R knockout mice and GIP induced insulin release is more sensitive to low levels of GIP than in wild type mice (Pederson et al., 1998). No increased sensitivity to glucagon with regards to insulin release was seen, nor is there
any change in glucagon mRNA in the pancreas or circulating glucagon levels in GLP-1 knockout mice (Scrocchi et al., 1998).

GLP-1 has been implicated in the function of neuroendocrine axes controlling reproduction, stress and fluid balance (Beak et al., 1996; Beak et al., 1998; Larsen et al., 1997b). GLP-1R knockout mice are reported to have normal circulating levels of testosterone, oestrogen and progesterone and reproduce successfully, although males have slightly reduced gonadal weights and pubertal onset is slightly delayed in females (MacLusky et al., 2000). Vasopressin levels and fluid balance are unaffected. Corticosterone levels are also normal despite a slight reduction in adrenal weight, however higher levels of corticosterone have been observed in GLP-1R knockout mice in response to stress suggesting some alteration to the stress response.

Heart rate and blood pressure are known to be increased in response to peripheral and central administration of GLP-1R agonists (Edwards et al., 1997; Barragan et al., 1994; Yamamoto et al., 2002). GLP-1Rs are expressed both in the heart (Wei and Mojsov, 1995) and in autonomic control centres in the brain (Alvarez et al., 1996; Merchenthaler et al., 1999) and thus could act at either, or both sites to affect cardiac function. GLP-1R knockout mice have been shown to have reduced resting heart rates and increased left ventricular end diastolic pressure compared to CD1 controls at 2 months of age and by 5 months old there is thickening of the left ventricular wall (Gros et al., 2003). This defect in diastolic function has not yet been shown to have severe functional consequences for the GLP-1R knockout mouse.
3.1.7 Chapter Summary and Aims

The experiments contained within this chapter aim to investigate and compare the mechanisms of glucagon, GLP-1 and oxyntomodulin-induced anorexia. Specifically, the following aims were addressed:

- The identification of receptors involved in mediating the anorectic effects of GLP-1, glucagon and oxyntomodulin
- The characterisation of the pattern of c-fos immunoreactivity seen in response to anorectic doses of glucagon and a comparison to that of GLP-1
- A comparison of the pattern of c-fos immunoreactivity induced by subanorectic doses of GLP-1 and glucagon when administered individually and in combination
- A direct comparison of the pattern of c-fos immunoreactivity induced by equivalently anorectic doses of glucagon, GLP-1 and oxyntomodulin and to identify by immunohistochemistry the populations of neurons in the NTS activated by these peptides.
3.2 MATERIALS AND METHODS

3.2.1 Animals

All animal procedures undertaken were approved by the British Home Office under the UK Animal (Scientific Procedures) Act 1986 (Project license 70/6402 for experimental work and 70/7096 for breeding). Male C57Bl/6 mice (Harlan, UK) or GLP-1R knockout mice (3.2.1.1) were housed in individually ventilated cages under controlled temperature (21-23°C) and a 12:12 hour light-dark cycle (lights on 0700). Animals had ad libitum access to food (RM1 diet, Special Diet Services Ltd, Witham, UK) and water unless otherwise stated.

3.2.1.1 GLP-1 Receptor Knockout mice

GLP-1R knockout mice were bred with the kind permission of Dr Daniel Drucker. The mice were bred from heterozygous knockouts. Studies using these mice used wild type littermates as controls. Genotyping was carried out by PCR using primers for the neomycin resistance gene, which forms part of the cassette replacing the exons coding for the first and third transmembrane domain of the receptor, and a second sequence which is removed in the KO animals. PCR reactions were carried out using the Phire Animal Tissue Direct PCR Kit according to the dilution protocol described by the manufacturers (Thermo Fisher Scientific, Finland). 1% DMSO and Betaine (Q solution, Qiagen) were used as enhancers. Neo primers: Forward- CTT GGG TGG AGA GGC TAT TC, Reverse- AGG TGA GAT GAC AGG AGA TC, Tm 63°C, WT primers: Forward- TACACAATGGGGAGCCCCTA, Reverse- AAGTCATGGGTGTCTGGA, Tm 70°C.

3.2.2 Acute Feeding studies

These studies aimed to investigate which receptor/s mediates the effect of glucagon on feeding.

3.2.2.1 Procedure (excluding studies in GLP-1R knockout mice)

See description in 2.3.2.3.2. For studies using co-administration of peptides and a receptor antagonist, the antagonist was administered five minutes prior to the peptide. EX-9-39 was
administered at a dose of 25 μmol/kg, which was shown not in itself to have an effect on food intake, and to be capable of reducing the effect of a 100 nmol/kg dose of GLP-1 on food intake (appendix 6.3). The glucagon receptor antagonist used (referred to as GCGR antag) was des-His-Glu9-glucagon and was administered at 7.5umol/kg in a solution of saline plus NaOH to bring it to pH 10, which was required for the peptide to dissolve.

3.2.2.2 Procedure (GLP-1R knockout mice and wild type littersmates)

Study protocol was as for acute feeding studies above, but studies used a cross-over within-subjects design (this was due both to the small number of animals available and to the greater variation in age and weight compared to wild type cohorts purchased from commercial breeders). Animals were randomised to a treatment group and each animal received all treatments across several studies. The order in which treatments were administered was random. Studies were carried out at four day intervals. Peptides were administered at the following doses: EX-4 5 nmol/kg, PYY3-36 200 nmol/kg, glucagon 1500 nmol/kg, oxyntomodulin 1000 nmol/kg.

3.2.3 c-fos immunohistochemistry studies in mice

The pattern of c-fos immunoreactivity induced by glucagon has not previously been investigated, and thus was carried out at doses ranging from the subanorectic (30 nmol/kg) to strongly anorectic (750 nmol/kg) to investigate the mechanisms by which this hormone affects feeding. A similar experiment was carried out using GLP-1 (3-600 nmol/kg) for comparison. A combination of subanorectic doses of GLP-1 and glucagon was found to reduce food intake (2.4.2.5). The pattern of c-fos immunoreactivity induced by this combination of hormones was thus compared to that of the individual, subanorectic doses. Finally a comparison of the pattern of c-fos immunoreactivity induced by equivalently anorectic doses was carried out to investigate whether any differences in their anorectic mechanisms were detectable.
3.2.3.1 Animal conditions and tissue preparation

The mice were handled daily and acclimatised to receiving s/c injections of saline over the two weeks prior to each study. Experiments were carried out during the early light phase (0800-1000) after fasting from 1600 the preceding day. Mice (n=6 per group) were injected s/c and immediately returned to their cages. Ninety minutes later, they were anaesthetised with intraperitoneal pentobarbitone (Euthatal, Merial Animal Health Ltd, Harlow, UK). The pedal reflex test was used to ensure that a surgical plane of anaesthesia had been reached. The mouse was placed in a dorsal recumbent position and a transverse incision was made just caudal to the xiphoid process. The diaphragm was incised, followed by the left and right lateral ribcage. The xiphoid process was clamped and held rostrally to expose the chest cavity. An 18-gauge needle, connected to a two-way valve was then inserted into the left ventricle and clamped into position. A small nick was made in the right atrium and the animal was perfused with from a 1m height under gravitational pressure. The animal was first flushed with 20-30 ml of flush solution followed by 5 minutes of fixation using fixative solution. Perfused brains were left in situ for four hours, cut coronally beneath the circle of Willis then post-fixed in the same fixative for 24 hours at 4°C. Cryoprotection was carried out in 30% sucrose/0.01M PBS before the brains were frozen in liquid nitrogen-cooled isopentane and stored at -80°C. Coronal sections through the brainstem and hypothalamus were cut at 40 μm using a sled microtome. Free floating sections were stored in antifreeze at -20°C.

3.2.3.2 c-fos immunohistochemistry

All stages were carried out at room temperature. Wash steps used 0.01M PBS prior to primary antibody application and 0.01M PBS with 0.05% Tween20 (VWR, Lutterworth, UK) (PBST) after this point. All solutions were made up in 0.01M PBS unless stated otherwise. Sections were washed to remove antifreeze before being transferred to a solution of 0.6% H₂O₂ (Sigma-Aldrich, Dorset UK) in methanol (VWR) for 15 minutes to quench endogenous peroxidise activity. The sections were then washed and incubated in a blocking solution (3% goat serum (Invitrogen) with 0.25% Triton X (Sigma-Aldrich)) for 2 hours to reduce non-specific binding of the antibody. The sections were then
incubated overnight with rabbit anti c-fos antibody (Merck Chemicals Ltd, Nottingham, UK) at a concentration of 1:20000 in blocking solution.

The sections were washed and incubated with secondary antibody solution (0.25% biotinylated goat anti-rabbit IgG (Vector Laboratories, UK), 0.3% w/v BSA and 0.25% Triton X) for two hours. They were then washed and incubated for 1 hour in ABC solution (Vector Laboratories), made using 1:200 dilution of avidin and biotin. This solution was made up 30 minutes before use to allow the avidin and biotin to form a complex. After a final wash, the antigen-antibody complex was visualised by incubation in 0.01M PBS containing 0.05% 3,3’-Diaminobenzidine tetrahydrochloride hydrate solution (DAB) (Sigma-Aldrich) and 0.03% hydrogen peroxide. The sections were removed from the DAB solution once the staining was sufficient in the appropriate sections of a positive control. The sections were then mounted onto polylysine coated slides (VWR) and left to dry overnight. Slides were dehydrated in increasing concentrations of ethanol (VWR) solutions in water (50% to 100% in three steps) and then dipped in xylene (VWR) before being mounted with DPX mountant (VWR) and coverslips.

3.2.3.3 Analysis

Total numbers of cell bodies positive for c-fos-like immunoreactivity were counted bilaterally from matched sections by a blinded observer in hypothalamic and brainstem nuclei known to be involved in appetite regulation and the central nucleus of the amygdala (CeA). Animals were only included in the analysis if they could be matched at all the chosen sections across a nucleus. Only darkly labelled oval-shaped nuclei were counted as c-fos positive. Regions of interest and nuclear boundaries were defined in relation to anatomical landmarks using a mouse brain atlas for guidance (Franklin and Paxinos, 1997). Data are presented as the mean and standard error of the mean number of c-fos positive nuclei across a nucleus. The number of matched sections counted for each nucleus in each study and the coordinates these sections were taken from, as defined in a mouse brain atlas (Franklin and Paxinos, 1997), are given in appendix 6.4.
3.2.4 Dual Immunohistochemistry for c-fos and markers of neuronal populations in the NTS

Dual immunohistochemistry was carried out to detect the presence of c-fos in neurons expressing either tyrosine hydroxylase (catecholaminergic), GLP-1 (PPG), ACTH (POMC) or CART, four populations of neurons found in the subpostremal and caudal portions of the NTS where glucagon, GLP-1 and oxyntomodulin appeared to increase c-fos expression. The PPG neurons were of particular interest as the results of feeding studies using EX-9-39 and in the GLP-1R knockout mice suggest a role for GLP-1R signalling in glucagon-mediated inhibition of food intake.

Immunohistochemistry for c-fos was carried out first and was performed as described in section 3.2.3.2 but with slight modifications. An avidin/biotin blocking step was used to block staining due to endogenous biotin which is present in certain cells in the CNS (McKay et al., 2004), and which could lead to increased background staining. Reduction of background staining is important for the detection of neuropeptides and other markers of neuronal populations, which are often more difficult to detect than c-fos. The Avidin/Biotin block was performed for 30 minutes after the primary antibody incubation (Vector labs). In addition, when staining for c-fos 1% NiCl was added to the DABs solution giving the precipitate a blue/black colour.

After the sections had been stained for c-fos they were stained subsequently for a second antigen. Antigen retrieval was carried out for 30 minutes at 90°C in 20mM Tris pH 9. This step improves the ability of many antibodies to bind to their antigens and thus increases their sensitivity. The procedure was then carried out as previously described, using a primary antibody against the desired antigen. The anti-GLP-1 antibody was used at 1:100 000 dilution (Peninsula Laboratories, San Carlos, CA), anti-TH was used at 1:75 000 (Calbiochem, San Diego, California), anti-CART was raised in house and used at 1:100 000 and anti-ACTH was used at 1:5000 (National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA ).
Due to the difficulty of IHC staining for ACTH in the brainstem a polymer based detection method was used in place of a secondary antibody/ABC system (ImmPRESS, Vector laboratories). This system uses a secondary antibody already conjugated to horseradish peroxidise. This method reduces background staining, as it is unaffected by endogenous biotin. It also produces a larger amplification of the signal compared to ABC detection methods. In addition, I found the inclusion of triton X in the blocking solutions and antibody diluents decreased the ACTH signal and thus triton X was not used during c-fos or ACTH staining in these sections. To compensate for the reduced cell permeability in sections without triton X treatment incubation with the ACTH antibody was carried out for 48 hours. DAB solution without nickel added was used as the chromagen to visualise the second antigen which creates a brown colour when oxidised.

### 3.2.5 Statistical analysis

Food intake and c-fos immunoreactivity in dose response studies was analysed using one-way ANOVA with Dunnett’s post hoc test comparing each group to the saline control. In the combined GLP-1/glucagon c-fos study data were analysed using one-way ANOVA followed by the Bonferroni post-hoc test, which allows planned comparisons between selected groups, to compare each group to saline and to compare the individual administered peptide groups to the co-administered peptide group. In the c-fos study comparing GLP-1, glucagon and oxyntomodulin data were analysed using one-way ANOVA followed by Tukey’s post-hoc test comparing all groups to one another (Prism v5, GraphPad Software Inc. San Diego, USA). In cross-over studies, comparing knockout to wild type animals, data were analysed using a two way ANOVA with genotype as the between-subjects variable and treatment as the within-subjects variable followed by the Bonferroni post hoc test (SPSS statistics v19, SPSS IBM, Chicago, IL). In all cases p<0.05 was considered statistically significant.
3.3 RESULTS

3.3.1 Effect of GLP-1 receptor antagonist on the anorectic effect of glucagon

The administration of EX-9 alone had no effect on food intake whereas glucagon reduced food intake significantly during the first 30 minutes after administration (Figure 3.5 A). When animals were pre treated with EX-9 however there was no significant reduction in food intake in response to glucagon, with a significant difference seen in the food intake of mice treated with EX-9 and glucagon compared to glucagon alone.

3.3.2 Effect of a glucagon receptor antagonist on the anorectic effect of glucagon

The administration of the glucagon receptor antagonist alone had a significant effect on food intake (Figure 3.5 B). Glucagon also reduced food intake significantly during the first 30 minutes after administration. When glucagon was administered to animals pre-treated with the glucagon receptor antagonist there remained a significant reduction in food intake compared to saline control and no significant difference in food intake compared to mice treated with glucagon alone.
Figure 3.5: Effect of GLP-1 and glucagon receptor antagonists on the anorectic effect of glucagon. 0-30 minute food intake (EX-9, GCGR antagonist or saline administered at t=-5, glucagon or saline administered at t=0. EX-9 25 μmol/kg, GCGR antag 7.5 μmol/kg, Glucagon 750 nmol/kg. Mice were fasted overnight, injected at the onset of the light phase s/c and given a measured amount of food. Food was reweighed at the specified times. N=7 *p<0.05, ** p<0.01 ***p<0.001. Average weight of mice 24.3g
3.3.3 Effect of EX-4, PYY\textsubscript{3-36}, glucagon (GCG) and oxyntomodulin (OXM) on food intake in GLP-1R knockout mice

This study aimed to compare the effect of the four peptide treatments in GLP-1R knockout and wild type animals. A two-way ANOVA showed a significant main effect of treatment and a significant genotype*treatment interaction effect at the 30 minute timepoint. Post hoc comparisons showed a significant effect of all four peptides at the 30 minute timepoint (p<0.001 EX-4, GCG and OXM, p<0.01 for PYY\textsubscript{3-36}) and that EX-4, glucagon and oxyntomodulin reduced food intake to a greater extent in wild type than in GLP-1R knockout animals at the 30 minute timepoint (p<0.001 EX-4 and GCG, p<0.01 OXM). At the 90 minute timepoint a two-way ANOVA showed a significant main effect of treatment and a significant genotype*treatment interaction effect. Post hoc analysis showed there was a significant effect of treatment with EX-4, glucagon and PYY\textsubscript{3-36} (p<0.01 EX-4 and PYY\textsubscript{3-36}, p<0.05 glucagon) and a significant treatment*genotype interaction effect in response to EX-4 (p<0.01). At the 180 minute timepoint a two-way ANOVA showed a significant main effect of treatment and a significant genotype*treatment interaction effect. Post hoc analysis showed there was a significant effect of treatment with EX-4, oxyntomodulin and PYY\textsubscript{3-36} (p<0.05 EX-4 and oxyntomodulin, p<0.01 PYY\textsubscript{3-36}) and a significant genotype-treatment interaction effect in response to EX-4 (p<0.05). There was no effect of genotype on the response to PYY\textsubscript{3-36} at any timepoint.
Figure 3.6: Effect of EX-4, PYY$_{3-36}$, glucagon and oxyntomodulin on food intake in GLP-1R knockout mice.

The mean difference in food intake between animals when administered with a peptide or saline control is shown. EX-4 5 nmol/kg, PYY$_{3-36}$, oxyntomodulin 1000 nmol/kg, glucagon 1500 nmol/kg. Open bars wild type littermates, hashed bars GLP-1R knockouts. A: 0-30 minutes, B: 30-90 minutes, C: 90-180 minutes. Mice were fasted overnight, injected at the onset of the light phase s/c and given a measured amount of food. Food was reweighed at the specified times. N=8-9 *p<0.05, ** p<0.01 ***p<0.001. Average weight 24.0g.
3.3.4 The effect of anorectic doses of glucagon on c-fos immunoreactivity

The effect of a range of doses of glucagon on food intake was examined in section 2.4.2.1 and the effects of the doses used in the c-fos study on feeding are reproduced in Figure 3.7 A.

Glucagon increased c-fos immunoreactivity in the area postrema at 300nmol/kg and higher doses (mean c-fos counts: AP: 7 ± 6 [saline] vs. 99 ± 17 [glucagon 300 nmol/kg], p<0.01 vs. saline, n=3-5/group; Figure 3.7 B). In the caudal nucleus tractus solitarius (cNTS), glucagon also significantly increased c-fos immunoreactivity at 300nmol/kg and higher doses (mean c-fos counts: cNTS: 3 ± 1 [saline] vs. 75 ± 4 [glucagon 300 nmol/kg], p<0.01 vs. saline, n=3-5/group; Figure 3.7 C). The same doses of glucagon increased c-fos immunoreactivity in the CeA, (mean c-fos counts: CeA: 28 ± 8 [saline] vs. 92 ± 15 [glucagon 300 nmol/kg], p<0.05 vs. saline, n=3-5/group; Figure 3.7 E). Only the 500nmol/kg dose significantly increased c-fos immunoreactivity in the parabrachial nucleus (PBN) (mean c-fos counts: PBN: 16 ± 1 [saline] vs 63 ± 10 [glucagon 500nmol/kg], p<0.01 vs. saline, n=5/group, Figure 3.7 D). Figure 3.8 shows representative images of c-fos immunoreactivity in response to saline or 750nmol/kg glucagon in the AP, NTS, PBN and CeA.

Glucagon did not significantly affect c-fos immunoreactivity in the hypothalamic arcuate (ARC), paraventricular (PVN), ventromedial (VMN) or dorsomedial hypothalamic nuclei (DMN) (Table 3.1).
Figure 3.7: The effects of glucagon on food intake and c-fos immunoreactivity in fasted mice. Saline (control) or glucagon (10, 30, 100, 300, 500, 750 nmol/kg) was injected s/c into overnight fasted mice in the early light phase. a: Food intake 0-30 minutes after injection. b-e: c-fos immunoreactivity in matched sections from brains fixed 90 minutes after injection, graphs show median, 25th and 75th percentile and range. b: area postrema (AP), c: nucleus tractus solitarius (NTS), d: lateral parabrachial nucleus e: central nucleus of the amygdala (CeA). ***p<0.001, **p<0.01, *p<0.05. Average mouse weight 27g.
Figure 3.8: Representative photomicrographs of c-fos immunoreactivity 90 minutes after subcutaneous saline or glucagon (750 nmol/kg) administration. a: saline NTS and AP; b: glucagon NTS and AP, c: saline lateral PBN, d: glucagon lateral PBN; e: saline CeA f: glucagon CeA. scale bar = 100μm
### Table 3.1: c-fos in the hypothalamus in response to a range of doses of glucagon.

<table>
<thead>
<tr>
<th>Nucleus/Region of interest</th>
<th>Dose (nmol/kg)</th>
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<th>50</th>
<th>100</th>
<th>200</th>
<th>500</th>
<th>750</th>
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<tbody>
<tr>
<td>ARC</td>
<td></td>
<td>62 ± 28</td>
<td>21 ± 4</td>
<td>61 ± 21</td>
<td>26 ± 8</td>
<td>40 ± 6</td>
<td>45 ± 19</td>
<td></td>
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<tr>
<td>PVN</td>
<td></td>
<td>28 ± 17</td>
<td>19 ± 2</td>
<td>22 ± 5</td>
<td>24 ± 13</td>
<td>18 ± 3</td>
<td>37 ± 7</td>
<td></td>
</tr>
<tr>
<td>VMN</td>
<td></td>
<td>9 ± 2</td>
<td>12 ± 5</td>
<td>24 ± 10</td>
<td>14 ± 5</td>
<td>8 ± 2</td>
<td>12 ± 2</td>
<td></td>
</tr>
<tr>
<td>DMN</td>
<td></td>
<td>40 ± 7</td>
<td>48 ± 21</td>
<td>64 ± 17</td>
<td>44 ± 14</td>
<td>83 ± 35</td>
<td>40 ± 13</td>
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</tbody>
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### Table 3.2: c-fos in the hypothalamus in response to a range of doses of GLP-1.

<table>
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<th>Nucleus/Region of interest</th>
<th>Dose (nmol/kg)</th>
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<th>30</th>
<th>50</th>
<th>100</th>
<th>600</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARC</td>
<td></td>
<td>15 ± 5</td>
<td>13 ± 4</td>
<td>13 ± 4</td>
<td>41 ± 16</td>
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<tr>
<td>PVN</td>
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<td>14 ± 5</td>
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<td>37 ± 3</td>
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<td>VMN</td>
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<td>16 ± 4</td>
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<td>16 ± 3</td>
<td>15 ± 2</td>
<td>17 ± 4</td>
<td>12 ± 6</td>
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<tr>
<td>DMN</td>
<td></td>
<td>27 ± 8</td>
<td>32 ± 7</td>
<td>21 ± 4</td>
<td>25 ± 6</td>
<td>23 ± 4</td>
<td>23 ± 4</td>
</tr>
</tbody>
</table>
3.3.5 The effect of anorectic doses of GLP-1 on c-fos immunoreactivity

The effect of a range of doses of GLP-1 on food intake was examined in section 2.4.2.2 and the effects of the doses used in the c-fos study on feeding are reproduced in Figure 3.9 A.

GLP-1 increased c-fos immunoreactivity in the AP at 50 nmol/kg and higher doses (mean c-fos counts: 6 ± 2 [saline] vs. 87 ± 14 [GLP-1 50 nmol/kg], p<0.001 vs. saline, n=3-5/group; Figure 3.9 B) and at doses of 30 nmol/kg and greater in the cNTS (mean c-fos counts: 19 ± 6 [saline] vs. 126 ± 17 [GLP-1 30 nmol/kg], p<0.05 vs. saline, n=3-5/group; Figure 3.9 C). GLP-1 induced statistically significant c-fos expression in the CeA at 50 nmol/kg and higher doses (mean c-fos counts: 28 ± 7 [saline] vs. 114 ± 16 [GLP-1 50 nmol/kg], p<0.01 vs. saline, n=4-5/group; Figure 3.9 E). Only the 100 nmol/kg dose significantly increased c-fos immunoreactivity in the parabrachial nucleus (PBN) (mean c-fos counts: 53 ± 13 [saline] vs. 104 ± 16 [GLP-1 100 nmol/kg], p<0.05 vs. saline, n=4-5/group; Figure 3.9 D).

GLP-1 did not significantly affect c-fos immunoreactivity in the hypothalamic Arc, PVN, VMH, or DMH (Table 3.2).
Figure 3.9: The effects of GLP-1 on food intake and c-fos immunoreactivity in fasted mice. Saline (control) or GLP-1 (10, 30, 50, 100, 300, 600 nmol/kg) was injected subcutaneously into overnight fasted mice in the early light phase. a: Food intake 0-30 minutes after injection. b-e: c-fos immunoreactivity in matched sections from brains fixed 90 minutes after injection, graphs show median, 25th and 75th percentile and range. b: area postrema (AP), c: nucleus tractus solitarius (NTS), d: lateral parabrachial nucleus e: central nucleus of the amygdala (CeA). ***p<0.001, **p<0.01, *p<0.05. Average weight 27.2g.
3.3.6 The effect of combined administration of GLP-1 and glucagon on c-fos immunoreactivity

The effect of combined administration of GLP-1 and glucagon on food intake was examined in section 2.4.2.5 and the effects of the doses used in the c-fos study on feeding are reproduced in Figure 3.10 A.

Administration of either hormone individually at these subanorectic doses did not significantly alter c-fos immunoreactivity in the AP, cNTS or PBN compared to saline controls. However, co-administration of the same doses of glucagon and GLP-1 significantly increased c-fos expression compared to saline in the AP (mean c-fos counts: 7 ± 4 [saline] vs. 46 ± 9 [co-administered GLP-1 and glucagon], p<0.05 vs. saline, n=5-6/group; Figure 3.10 B and Figure 3.11 D) and in the CeA (mean c-fos counts 75 ± 15 [saline] vs. 278 ± 33 [co-administered GLP-1 and glucagon], p<0.001 vs. saline, n=5-6/group; Figure 3.10 E and Figure 3.11 L). In addition, coadministration of GLP-1 and glucagon increased c-fos expression in the CeA to a greater extent than either hormone individually (mean c-fos counts 175 ± 24 [GLP-1], 88 ± 17 [glucagon], p<0.05 GLP-1 vs. coadministered GLP-1 and glucagon, p<0.01 glucagon vs. coadministered GLP-1 and glucagon, n=5-6/group). Coadministration of GLP-1 and glucagon did not significantly affect c-fos expression in the PBN and cNTS.
Figure 3.10: The effects of coadministration of GLP-1 and glucagon on food intake and c-fos immunoreactivity in fasted mice. Two simultaneous injections of saline/saline (control), saline/GLP-1 10 nmol/kg, saline/glucagon 30 nmol/kg or GLP-1/glucagon (10 nmol/kg /30 nmol/kg) were administered subcutaneously to overnight fasted mice in the early light phase. a: Food intake 0-30 minutes after injection. b-e: c-fos immunoreactivity in matched sections from brains fixed 90 minutes after injection of saline vs. 10 nmol/kg GLP-1, 30 nmol/kg glucagon or 10 nmol/kg GLP-1 and 30 nmol/kg glucagon. b: area postrema (AP), c: nucleus tractus solitarius (NTS), d: lateral parabrachial nucleus e: central nucleus of the amygdala (CeA). ***p<0.001, **p<0.01, *p<0.05. *compared to saline, ^GCG compared to GLP-1+GCG, $GLP-1 compared to GLP-1+GCG. Mean weight 28.6g
Figure 3.11: Representative photomicrographs of c-fos immunoreactivity 90 minutes after subcutaneous administration of saline, GLP-1, glucagon or GLP-1 + glucagon. a-d: NTS and AP; e-h: lateral parabrachial nucleus; i-l: CeA. a: Saline b: GLP-1 (10nmol/kg) c: Glucagon (30nmol/kg) d: GLP-1 (10 nmol/kg) + glucagon (30 nmol/kg) e: Saline f: GLP-1 (10nmol/kg) g: Glucagon (30nmol/kg) h: GLP-1 (10 nmol/kg) + glucagon (30 nmol/kg) i: Saline j: GLP-1 (10nmol/kg) k: Glucagon (30nmol/kg) l: GLP-1 (10 nmol/kg) + glucagon (30 nmol/kg). scale bar = 100μm
3.3.7 Comparison of effects of GLP-1, glucagon and OXM on c-fos expression

3.3.7.1 Dose finding

This study aimed to find doses of GLP-1, glucagon and OXM which had a potent, but submaximal, effect on food intake at the 0-30 minute timepoint and which reduced food intake to the same degree. The administration of the 500 nmol/kg GLP-1, 1500 nmol/kg glucagon and 1000 nmol/kg of oxyntomodulin had a significant effect on food intake at 30 minutes (Figure 3.12). All three peptides reduced food intake to a similar extent at these doses.

![0-30 minute food intake graph](image)

Figure 3.12: Dose finding for comparison of effects of GLP-1, glucagon and OXM on c-fos expression. GLP-1 500 nmol/kg, GCG 1500 nmol/kg, OXM 1000 nmol/kg. Mice were fasted overnight, injected at the onset of the light phase s/c and given a measured amount of food. Food was reweighed at 30 minutes post injection N=8 ***p<0.001. Mean mouse weight 22.2g
3.3.7.2 Comparison of effects of GLP-1, glucagon and OXM on c-fos expression

Administration of equivalently anorectic doses of GLP-1, glucagon or OXM did not increase c-fos expression significantly in the AP compared to saline controls (Figure 3.13 A). In the caudal NTS (comprised of the subpostremal NTS and NTS adjacent to the obex), OXM increased c-fos expression significantly compared to saline and compared to either GLP-1 or glucagon (mean c-fos counts: 43 ± 10 [saline], 96 ± 24 [GLP-1], 116 ± 12 [GCG], 233 ± 20 [OXM], p<0.001 OXM vs. saline, p<0.001 OXM vs. GLP-1, p<0.01 OXM vs. GCG, n=4-5/group; Figure 3.13 B). When the subpostremal NTS was considered separately, OXM still increased c-fos immunoreactivity when compared to saline but there was no statistically significant difference between c-fos like immunoreactivity after administration of OXM compared to GLP-1 or glucagon (mean c-fos counts: 4 ± 2 [saline], 83 ± 17 [OXM], p<0.01 OXM vs. saline, 4-5/group; Figure 3.13 C). In the NTS immediately adjacent to the obex, OXM increased c-fos expression significantly compared to saline and compared to either GLP-1 or glucagon (mean c-fos counts: 39 ± 8 [saline], 63 ± 16 [GLP-1], 60 ± 9 [GCG], 137 ± 9 [OXM], p<0.001 OXM vs. saline, p<0.01 OXM vs. GLP-1, p<0.01 OXM vs. GCG, n=4-5/group; Figure 3.13 D).

None of the peptides caused a significant increase in c-fos expression in the parabrachial nucleus. In the CeA OXM increased c-fos expression compared to saline, but was not significantly different to GLP-1 or glucagon administration (mean c-fos counts 24 ± 11 [saline], 102 ± 11 [OXM], p<0.05 vs. saline, n=5-6/group; Figure 3.13 E). None of the peptides caused a statistically significant increase in c-fos expression in any of the hypothalamic nuclei assessed, although there was perhaps a trend towards significant c-fos induction by oxyntomodulin in the ARC (mean c-fos counts 16 ± 6 [saline], 37 ± 10 [saline], p=0.22) and PVN (mean c-fos counts 35 ± 10 [saline], 68 ± 14 [OXM], p=0.28) (Table 3.3).
Figure 3.13: Comparison of effects of GLP-1, glucagon and OXM on c-fos expression. Injections of saline (control), GLP-1 500 nmol/kg, glucagon 1500 nmol/kg or OXM (1000 nmol/kg) were administered subcutaneously to overnight fasted mice in the early light phase. Graphs show mean and SEM of c-fos immunoreactivity in matched sections from brains fixed 90 minutes after injection a: area postrema (AP), b: caudal nucleus tractus solitarius (cNTS), c: subpostremal nucleus tractus solitarius (spNTS) d: nucleus tractus solitarius adjacent to the obex (aNTS) e: parabrachial nucleus f: central nucleus of the amygdala (CeA). ***p<0.001, **p<0.01, *p<0.05. *compared to saline, #compared to GLP-1, $compared to glucagon. Mean mouse weight 24.0g.
<table>
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<tr>
<th>Nucleus/Region of interest</th>
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<td>Saline</td>
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<td>ARC</td>
<td>16 ± 6</td>
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<td>PVN</td>
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<td>VMH</td>
<td>18 ± 4</td>
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<td>DMH</td>
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Table 3.3: c-fos in the hypothalamus in response to GLP-1, glucagon or OXM.
3.3.8 Investigation of neuronal populations in the NTS activated by GLP-1, glucagon and oxyntomodulin

3.3.8.1 TH expressing neurons

Immunohistochemical staining for c-fos and TH was examined throughout the caudal and subpostremal portions of the NTS. In the caudal NTS, (Figure 3.14 + Figure 3.15) occasional co-localisation of c-fos and TH staining was observed in response to GLP-1, glucagon and oxyntomodulin, with no obvious difference in the pattern or quantity of colocalised staining. In the saline treated animals there was also occasional co-localisation of c-fos and TH staining, although this did appear to occur less frequently than in sections from peptide treated animals.

In the subpostremal portion of the NTS, where c-fos staining was more intense, colocalisation of c-fos and TH immunoreactivity was also seen in occasional TH expressing neurons in all three peptide treated groups. Figure 3.16 shows the dorsomedial portion of the NTS, adjacent to the AP, where a number of TH immunopositive neurons were localised along the edge of the AP. These neurons rarely showed co-localisation with c-fos immunoreactivity, although occasional neurons were c-fos positive in the oxyntomodulin treated group. In the ventrolateral portion of the NTS colocalisation of c-fos and TH immunoreactivity was commonly seen in all four groups (Figure 3.17).
Figure 3.14: Representative photomicrographs of immunohistochemical staining for c-fos and TH in the caudal NTS. c-fos in blue/black, TH in brown. A: saline, B: GLP-1, C: glucagon, D: oxyntomodulin. Sections located approximately at bregma -8.1 mm.
Figure 3.15: Representative photomicrographs of immunohistochemical staining for c-fos and TH in the caudal NTS. c-fos in blue/black, TH in brown. A: saline, B: GLP-1, C: glucagon, D: oxyntomodulin. Sections located approximately at bregma -7.9 mm.

Figure 3.16: Representative photomicrographs of immunohistochemical staining for c-fos and TH in the subpostremal NTS. c-fos in blue/black, TH in brown. A: saline, B: GLP-1, C: glucagon, D: oxyntomodulin. Sections located approximately at bregma -7.5 mm, adjacent to the AP.
Figure 3.17: Representative photomicrographs of immunohistochemical staining for c-fos and TH in subpostremal NTS. c-fos in blue/black, TH in brown. A: saline, B: GLP-1, C: glucagon, D: oxyntomodulin. Sections located approximately at bregma -7.5 mm, in the ventrolateral portion of the NTS.
3.3.8.2 ACTH expressing neurons

ACTH immunoreactivity was detected in the caudal and subpostremal portions of the NTS, although the immunoreactivity did not appear as strongly in cell bodies as in hypothalamic sections (appendix 6.5). ACTH immunoreactive neurons were more sparsely distributed than those expressing TH, and in the subpostremal NTS, were located exclusively in the lateral portions. Co-localisation of c-fos with ACTH immunoreactivity was seen in a few neurons in the caudal NTS in sections from animals treated with GLP-1, glucagon and oxyntomodulin, but not in saline controls (Figure 3.18). In the subpostremal NTS co-localisation of c-fos and ACTH immunoreactivity was seen in sections from glucagon, GLP-1 and oxyntomodulin treated animals, and not in saline controls (Figure 3.19).

Figure 3.18: Representative photomicrographs of immunohistochemical staining for c-fos and ACTH in the caudal NTS. c-fos in blue/black, ACTH in brown. A: saline, B: GLP-1, C: glucagon, D: oxyntomodulin. Sections located approximately at bregma -8.1 mm.
Figure 3.19: Representative photomicrographs of immunohistochemical staining for c-fos and ACTH in the subpostremal NTS. c-fos in blue/black, ACTH in brown. A: saline, B: GLP-1, C: glucagon, D: oxyntomodulin. Sections located approximately at bregma -7.6 mm.
3.3.8.3 GLP-1 expressing neurons

GLP-1 immunoreactivity was detected in the caudal portion of the NTS, where they were seen in the lateral portion of the nucleus. There were no events of co-localisation of c-fos and GLP-1 immunoreactivity in any of the peptide treated groups or in the saline control animals. C-fos immunoreactivity appeared mostly medial to the GLP-1 expressing neurons (Figure 3.20).

Figure 3.20: Representative photomicrographs of immunohistochemical staining for c-fos and GLP-1 in the caudal NTS. c-fos in blue/black, GLP-1 in brown. A: saline, B: GLP-1, C: glucagon, D: oxyntomodulin. Sections located approximately at bregma -7.9 mm.
3.4 **DISCUSSION**

3.4.1 **The action of glucagon**

The anorectic effect of glucagon was attenuated by pre-treatment with the GLP-1R antagonist EX 9-39. Although there was no statistically significant difference between food intake in the EX 9-39 and glucagon coadministered group and the saline control group, there does appear to be some remaining inhibition of food intake. This may be due to insufficient blockade of the GLP-1Rs by EX 9-39 allowing some receptors to be activated. Alternatively it could indicate an anorectic effect of glucagon which does not depend on GLP-1R activation.

Pre-treatment with a glucagon receptor antagonist did not significantly alter the effect of glucagon on food intake. However the glucagon receptor antagonist reduced food intake significantly when administered by itself, making the results of this study difficult to interpret. This antagonist would only dissolve at low pH (pH 10) and as such the low pH may have caused some discomfort to the animals resulting in a reduction in food intake.

In global GLP-1R knockout mice, glucagon did not significantly reduce food intake compared to saline control animals, whereas in wild type littermates there was a significant reduction in food intake at 0-30 minutes after administration. Whilst neither EX-4 nor oxyntomodulin were anorectic in the GLP-1R knockout mice, PYY$_{3-36}$ did cause a significant reduction in food intake, showing that the appetite inhibitory pathways are intact in the GLP-1R knockout animals.

Taken together these studies suggest the anorectic effect of glucagon is dependent on the presence of functional GLP-1Rs and that their pharmacological blockade inhibits glucagon’s anorectic effect. There are several possible explanations for these findings. The first is that glucagon inhibits feeding by cross reaction with the GLP-1R. Glucagon binds to the GLP-1R with low affinity, with and IC$_{50}$ of approximately 1µM (Thorens, 1992), which may suggest this explanation is unlikely, but cannot be ruled out from the current studies. A second explanation is that GLP-1R activation occurs downstream of glucagon binding to the glucagon receptor. As a result the blockade or absence of
functional GLP-1Rs could still prohibit an anorectic effect of glucagon. The investigation of c-fos expression in PPG neurons in the brainstem after glucagon administration was investigated to address this (see section 3.4.5.1.1). A third possibility is that glucagon is only anorectic in the presence of endogenous GLP-1 which activates the GLP-1R. It has been shown that glucagon is not anorectic in sham fed rats suggesting that some factor associated with ingestion of nutrients is required for glucagon to inhibit feeding (Geary and Smith, 1982a). As GLP-1 is released post-prandially it is possible this factor is GLP-1 and that concurrent activation of glucagon and GLP-1Rs is necessary to observe an anorectic effect in response to glucagon administration. If this were the case however it would be expected that a glucagon receptor antagonist would also inhibit the anorectic effect of glucagon, which did not appear to be the case in the current study, although the validity of the glucagon receptor antagonist study is questionable as discussed above.

3.4.2 The activation of central appetite regulating centres by glucagon

To investigate the possible mechanisms of glucagon-induced reduction in food intake, c-fos studies were undertaken to determine the regions of the hypothalamus and brainstem in which neuronal activation occurs after glucagon administration. In the brainstem, increased c-fos immunoreactivity was observed in the AP and NTS in response to glucagon at doses of 300nmol/kg and above. Lesions of both these areas have been previously shown to prevent glucagon’s inhibition of food intake (Weatherford and Ritter, 1988), implying that the activation seen in these areas is required for inhibition of food intake. However whilst significant inhibition of food intake was evident at a dose of 100 nmol/kg this was not sufficient to induce significant c-fos expression in either the AP or the NTS. It is likely this apparent discrepancy is an artefact of comparing two very different experimental measures. Feeding studies are technically simple. As a result larger numbers of animals can be used and there is rarely a cause to exclude animals from the analysis. In contrast, in c-fos studies animals can be excluded for a number of reasons, either because of unsatisfactory perfusion-fixation, damage to sections during the slicing or staining process or an insufficient anatomical match between brain sections stained in a particular animal and those in the rest of the group. Thus overall...
the number of animals in each group in the c-fos studies is often fewer than in the feeding studies, reducing the statistical power of these experiments and their ability to detect small differences in neuronal activation. It can be seen from the graphs that glucagon is likely to increase c-fos expression in the AP and NTS at all anorectic doses tested.

The AP is thought to be involved in drug-induced nausea and emesis (Borison, 1989) and neurons in both the AP and NTS have been shown to be activated in response to emetic stimuli in the ferret (Boissonade et al., 1994). The induction of c-fos expression in these nuclei by glucagon raises the question of whether the food intake reduction seen in response to glucagon may be related to nausea. The study of nausea and emesis in rodents is difficult as they do not possess an emetic reflex. Conditioned taste aversion tests are often used to assess nausea in rodents and may help to determine whether the dose of glucagon used in these studies is capable of causing aversion. However the interpretation of these studies is difficult due to the ability of substances, such as amphetamines, to induce conditioned taste aversion despite also inducing self-administration in mice (Parker, 2003).

Nausea has been reported in humans given 1.5 mg glucagon infusions over a six hour period. However there was a delay in the nausea experienced by the volunteers: peak glucagon concentrations were reached at 30 minutes and yet nausea did not occur until 2.5-3 hours into the infusion (Ranganath et al., 1999). This implies glucagon triggered nausea by an indirect mechanism and the authors suggest an inhibition of nutrient passage through the proximal small intestine might be responsible. It would seem unlikely that this delayed effect is responsible for the acute inhibition of food intake seen in response to glucagon.
c-fos immunoreactivity in the PBN was studied in response to glucagon and GLP-1 because of the extensive projections this nucleus receives from the NTS (Herbert et al., 1990) and the activation seen in this nucleus in response to the GLP-1R agonist EX-4 in a previous study (Baraboi et al., 2011). c-fos expression in the lateral PBN was significantly increased by only one dose of glucagon in the current study, and not at the highest dose tested, which might suggest that activation of neurons in the PBN is not required to mediate the anorectic effects of glucagon, and may in fact be an anomaly. However the statistical analysis used for this data made adjustments for the number of comparisons made and thus the significant activation seen after 500nmol/kg of glucagon cannot be dismissed in this manner. It would be useful to repeat the study to determine whether the effect at the 500nmol/kg dose is reproducible.

A clear increase in c-fos expression was seen in the CeA in response to glucagon (although as was the case in the AP and NTS the doses required were higher than the lowest effective anorectic dose). Like the PBN, the CeA is involved in the response to palatable foods. This region was examined when looking at the effects of glucagon on neuronal activation both because it has previously been shown to be activated by GLP-1 (Baumgartner et al., 2010), and because it is part of the gustatory processing pathway involving the NTS and PBN which has been shown previously to be modulated by glucagon (Giza et al., 1993). The level of c-fos expression in the CeA in response to glucagon was more clearly dose-dependent than that in the PBN. There are direct projections from the NTS to the CeA (Reyes and Van Bockstaele, 2006) and the lack of c-fos expression induced in the PBN might suggest that signals ascend from the NTS to the CeA via these direct pathways rather than via the PBN. Immunohistochemistry for c-fos expression combined with neuronal tracers could be used to investigate this further.

It is not possible to determine from studies of neuronal activation whether glucagon is acting directly at receptors on neurons in activated areas of the brain, or whether glucagon binds at a distant site triggering a series of events leading to the central activation observed. Neither glucagon receptor
expression nor glucagon binding has been investigated in the brainstem. However hepatic vagotomy has been shown to prevent the effect of glucagon on food intake (Geary and Smith, 1983) and hepatic vagal afferents project to the NTS (Rogers and Hermann, 1983). The NTS activation seen in response to glucagon administration in the current study is consistent with the hypothesis that glucagon acts on receptors in the periphery and signals reach the brain via vagus inputs to the NTS.

The lack of any increase in c-fos expression in the hypothalamus in response to glucagon is perhaps surprising given the importance of the hypothalamus in appetite regulation. Strong hypothalamic c-fos staining in saline-treated animals was not evident (Table 3.1) hence it is unlikely that background activation masked an effect of glucagon in the hypothalamus. Whilst of undoubted importance in the regulation of food intake, the hypothalamus is not required for meal termination (Grill and Norgren, 1978a) and the brainstem is thought to be sufficient to mediate the anorectic effect of peripherally administered GLP-1 (Hayes et al., 2008). The lack of c-fos expression induced by glucagon in the hypothalamus suggests that the brainstem and amygdala are sufficient to mediate its anorectic effect. However, as discussed in section 3.1.6.2.4, c-fos expression is not a necessary consequence of neuronal activation and thus the possible involvement of hypothalamic centres in the anorectic effects of glucagon cannot be discounted.

3.4.3 The action of GLP-1 and a comparison to that of glucagon

The c-fos expression seen in the AP, NTS and CeA in response to GLP-1 in this study replicates published findings (Baumgartner et al., 2010). Previous studies have demonstrated c-fos expression in the ARC and PVN after peripheral GLP-1 administration (100 nmol/kg) (Abbott et al., 2005; Asarian, 2009). At a dose of 100 nmol/kg GLP-1, no statistically significant effect on ARC or PVN c-fos expression was seen in the current study, although there was a possible trend towards an increase in c-fos in the ARC at this dose. In the PVN there may also have been a trend towards small increase in c-fos expression at higher doses of GLP-1. The difference in dose of GLP-1 required to increase c-fos
expression in the ARC and PVN in this study compared to published studies may be due to a difference in route of administration. No published studies have used subcutaneous injections as were used in the current study. The pharmacokinetics of GLP-1 absorption from the subcutaneous and intraperitoneal routes appear to be very similar, and thus it is likely circulating levels of GLP-1 are similar after subcutaneous and intraperitoneal administration (Parkes, 2001). However the active fragment of CCK has been shown to have a greater effect on food intake when administered intraperitoneally, which is thought to be due to a paracrine effect on the intestine (Ebenezer, 1999). If GLP-1 were to have a similar effect this may explain the difference in neuronal activation seen in published studies compared to the current study.

No difference in the c-fos expression induced by GLP-1 and glucagon can be seen from these dose response studies. However as they are separate studies a direct comparison cannot be made. See section 3.4.5 for discussion on this point.

3.4.4 The action of combined GLP-1 and glucagon

A combination of GLP-1 and glucagon at doses too low to inhibit food intake individually reduced food intake when administered in combination (2.4.2.5). c-fos expression in response to combined administration of low doses of GLP-1 and glucagon (10 and 30 nmol/kg respectively) was examined. Administration of either hormone individually at these subanorectic doses did not significantly alter c-fos immunoreactivity in the AP, cNTS, PBN or CeA compared to saline controls. However, in the CeA coadministration of GLP-1 and glucagon increased c-fos counts significantly more than saline or either peptide administered individually. The increased activation seen only in the CeA after administration of both peptides compared to either peptide individually may suggest that this region is the most important for mediating the reduction of food intake. However this would be a very difficult inference to prove. A comparison of the effect of combined GLP-1 and glucagon administration in sham and CeA lesioned animals might indicate whether the CeA is necessary for
the effect of the combined peptides, but interpretation of lesion studies is limited by potential alterations in other aspects of the animals’ physiology and behaviour.

c-fos expression in the AP was increased compared to saline controls after combined administration of GLP-1 and glucagon. In contrast to the findings in the CeA, there was no significant difference between the effects of the individual peptides and the combined peptides. Thus whilst it is possible to say that combined administration of GLP-1 and glucagon at subthreshold doses induced c-fos expression in the AP, where doses of the individual peptides did not, it is not possible to claim that the combination of GLP-1 and glucagon caused any greater activation in this nucleus than the individual peptides. This finding must therefore be interpreted with caution as discussed in a recent review on the statistical analysis of combination treatment studies (Nieuwenhuis et al., 2011). The lack of increased c-fos expression in the NTS or PBN after combined administration of GLP-1 and glucagon might indicate these regions are less important for mediating the anorectic effect of this combination. However, although there is a dose dependent increase in c-fos expression in the AP and NTS in response to increasing doses of GLP-1 (Figure 3.9) and glucagon (Figure 3.7), the dose response is fairly shallow. This may mean the difference in anorectic effect is too small to show up as a difference in c-fos in these areas.

3.4.5 Comparison of effects of GLP-1, glucagon and OXM on c-fos expression

The aim of this study was to compare the effects of GLP-1, glucagon and oxyntomodulin on c-fos expression in the same study, and at equivalently anorectic doses. The doses used in this study were high, but submaximal for inhibiting fasting-induced food intake (Figure 3.12). It might be noted that the doses required to obtain this reduction in food intake are greater than doses causing a similar reduction in food intake in the dose response studies (Figure 2.6 and Figure 2.7). This is likely to be because the animals used were lighter, and thus as doses were calculated per kilo of body weight, the absolute amount of peptide administered to a lighter animal is less than for a heavier animal at the same dose. As the metabolism of an animal approximately scales to Mass$^{0.75}$ (Kleiber, 1947) this,
amongst other variables, may mean that in reality larger animals receive a greater effective dose of peptide and thus at the same dose in nmol/kg a peptide will be less effective in smaller animals.

In contrast to the dose response studies with GLP-1 and glucagon, neither peptide significantly increased c-fos expression in the area postrema, caudal NTS or CeA in this study. This is surprising given the strong inhibition of food intake seen in mice of the same average weight and under the same experimental conditions. However OXM did induce significant c-fos expression in the caudal NTS and the amount of c-fos expression seen was significantly different to that induced by either GLP-1 or glucagon. The difference in expression appears mainly to be derived from the portion of the NTS adjacent to the 4th ventricle, rather than in the subpostremal portion where there is no significant difference between the c-fos expression in the three peptide treated groups.

There are no known unique roles of the portion of the NTS adjacent to the 4th ventricle, however it has been shown to receive information from sensory neurons originating in the cecum (Altschuler et al., 1991). This region contains numerous neuronal subtypes, including catecholaminergic neurons and mineralocorticoid sensitive neurons which are thought to be involved in sodium appetite (Geerling et al., 2006). Projections have been observed from the CeA to this portion of the NTS (Danielsen et al., 1989).

The results of previous studies aiming to characterise the c-fos expression induced by oxyntomodulin have not been consistent with one another. Peripherally administered oxyntomodulin increased c-fos expression in the PVN, NTS and AP in one study, with no increase in c-fos expression in the ARC (Baggio et al., 2004b). In a separate study c-fos expression was elevated in the ARC, but not the PVN, NTS or AP, in response to peripherally administered oxyntomodulin (Dakin et al., 2004). The doses used in these studies were different, with Dakin et al. using less than one tenth of the dose used by Baggio et al., which may explain the discrepancy. Perhaps surprisingly, whilst Dakin et al. found their lower dose of oxyntomodulin to be anorectic when administered
intraperitoneally, Baggio et al. found their, ten-fold higher, dose was not anorectic by the same route.

In MEMRI studies oxyntomodulin appeared to reduce neuronal activation in the PVN, ARC and SON and increase activation in the VMH and AP, with no effect in the NTS (Chaudhri et al., 2006; Parkinson et al., 2009). Thus the results of these MEMRI studies do not correlate well with those of the c-fos expression studies. This too might be due to a difference in dose used, as Chaudhri et al. and Parkinson et al. used doses of oxyntomodulin 3-10 times higher than Baggio et al., and thus 30-100 times higher than Dakin et al. Alternatively the discrepancy may be accounted for by differences in what is being measured by the two methodologies. c-fos measures a binary response in individual neurons (expressed or not expressed over a threshold level) and MEMRI measurements reflect the uptake of manganese ions across a region of interest. Thus whilst some neurons are activated, and thus express c-fos, it is possible for the overall uptake of manganese ions to decrease, giving opposite results.
3.4.5.1 Investigation of neuronal populations in the NTS activated by glucagon, GLP-1 and oxyntomodulin

Of the four NTS neuronal populations I set out to investigate, I was able to identify three by immunohistochemistry. Whilst CART neurons were easily identified in the hypothalamus, this was more difficult in the brainstem as the peptide did not appear to be localised to the cell body. Of the populations I was able to identify, none appeared to account for a large number of the neurons expressing c-fos in response to GLP-1, glucagon or oxyntomodulin and no differences between the neurons activated by these three peptides could be seen.

3.4.5.1.1 PPG immunoreactive neurons

Neither GLP-1, glucagon nor oxyntomodulin appeared to activate any GLP-1 expressing neurons in the NTS. This finding has several implications. Firstly, as it appears peripherally administered glucagon does not activate the GLP-1 expressing neurons in the NTS, this would suggest the lack of an anorectic effect of glucagon in GLP-1R knockout mice, or in mice where a GLP-1R antagonist is co-administered, is not due to a blockade of signalling from these neurons. Thus the anorectic effect of glucagon must be due either to a direct reaction with the GLP-1R or be reliant on GLP-1 signalling elsewhere in the brain or periphery. Secondly both GLP-1 and glucagon are known to induce nausea and aversion (Ritzel et al., 1995; Colon et al., 1999), and the GLP-1 expressing neurons in the NTS are activated by aversive stimuli such as lithium chloride (Rinaman, 1999b). The fact that these neurons are not activated by any of the preproglucagon derived peptides tested suggests they may induce nausea and aversion by a different mechanism. Thirdly it has often been suggested that the reason for the similar effects of centrally and peripherally administered GLP-1 on appetite may be due to an activation of GLP-1 neurons in the NTS by vagal neurons which are in turn activated by peripherally released or administered GLP-1 (Williams, 2009; Larsen and Holst, 2005; Vrang and Larsen, 2010). The results of this study would suggest this is not the case.
3.4.5.1.2 TH-immunoreactive neurons

Occasional neurons expressing TH in the NTS co-expressed c-fos in response to glucagon, GLP-1 or oxyntomodulin. Although c-fos expression in TH-immunoreactive neurons in the NTS has not been investigated in response to glucagon, GLP-1 or oxyntomodulin previously, EX-4 has been shown to induce c-fos expression in these neurons (Yamamoto et al., 2002). However many of the TH-immunoreactive neurons which appear to be activated by these hormones, also show c-fos immunoreactivity in saline control sections (Figure 3.17), thus at least some of this activation should be attributed to non-specific, likely stress induced, artifacts. There is no information about the expression of c-fos in TH-immunoreactive neurons in saline treated animals in the publication by Yamamoto et al., but it seems likely that some of the c-fos expression in these neurons they attribute to EX-4 in this study may also be non-specific.

3.4.5.1.3 ACTH-immunoreactive neurons

Co-localisation of c-fos and ACTH immunoreactivity was seen in a few cells in response to glucagon, GLP-1 or oxyntomodulin, although not in the majority. This would suggest these may be involved in mediating the anorectic response of these peptides. ACTH positive processes were seen throughout the caudal and subpostremal NTS and many c-fos positive nuclei appeared to have appositions with ACTH expressing projections. Further work would be required to determine whether POMC expressing neurons might modulate the response of these neurons to glucagon, GLP-1 or oxyntomodulin.
3.4.6 Discussion of methodologies used and their limitations

3.4.6.1 Pharmacological blockade and genetic deletion of the GLP-1R

The use of the GLP-1R knockout mouse for experiments contained in this thesis has advantages over the use of a pharmacological blockade of the GLP-1R. Pharmacological antagonists may have off target effects thus reducing the specificity of a pharmacological blockade compared to genetic deletion. The half-life of pharmacological agents must be taken into account and experiments adjusted such that both antagonist and treatment are expected to act during the same time period.

The blockade of GLP-1 and glucagon-induced feeding by EX 9-39 suggest that the timing of antagonist administration was correct in these studies.

The phenotypic abnormalities of the GLP-1R knockout mouse, described in section 3.1.6.3.2, render genetic deletion of the GLP-1R a, perhaps equally, flawed model. The combination and comparison of both approaches can be used to an extent to circumvent these problems, but they must always be considered in the interpretation of results.

3.4.6.2 Dual immunohistochemistry for identification of c-fos expressing neurons

See section 3.1.6.2.4 for a discussion of the limitations of c-fos immunohistochemistry as a marker of neuronal activation.

The detection of GLP-1 expressing neurons by immunohistochemistry has been criticized in the past (Trapp and Hisadome, 2011; Llewellyn-Smith et al., 2011) for not detecting all the GLP-1 expressing neurons which are seen in the mouse model expressing yellow YFP under the PPG promoter. However, despite the difficulties these authors report, I found a very similar pattern of GLP-1 expression using immunohistochemistry for GLP-1 to that seen in the GLP-1-YFP mouse. It is possible that some neurons are not stained and that GLP-1, glucagon or oxyntomodulin may activate some of these. This seems unlikely however given the spatial separation of GLP-1 immunoreactivity and c-fos expression I observed.
Immunohistochemical detection of neuropeptides or other markers is intrinsically variable, and difficult to standardise. Although I am confident I selected appropriate antibody concentrations and detection methods there is no guarantee that, for any of the markers I used, all neurons expressing the markers were detected. Thus it remains possible that POMC, GLP-1 or TH expressing neurons were activated by the PPG peptides. Neuropeptides in particular are often exported away from the cell body, and if they cannot be detected surrounding a c-fos positive nucleus, that neuron is not deemed to be expressing the neuropeptide of interest in the current studies. For this reason mouse models expressing fluorescent or other markers under the control of PPG, POMC or CART promoters would be advantageous over immunohistochemical detection of the peptides themselves. Despite these limitations, strong GLP-1, ACTH and TH immunoreactivity was visible in cell bodies within the NTS in the current studies, and thus the lack of co-expression of c-fos in these neurons in response to the peptides tested can be said to suggest they are not major downstream targets of the PPG-derived peptides.
3.4.7 Conclusions and future directions

Glucagon, GLP-1 and oxyntomodulin behaved very similarly in the experiments within this chapter. It would appear that the anorectic effects of all three are due to, or at least dependant on activation of, the GLP-1R. The patterns of c-fos expression seen in response to glucagon and GLP-1 appeared very similar, and the dose-response relationships largely paralleled those seen in feeding studies in section 2.4.2. When both peptides were combined at doses subthreshold for inhibition food intake, c-fos expression was induced in the amygdala, to a greater extent than was seen when either peptide was administered individually. This may illustrate that the amygdala plays an important role in mediating the anorectic effect seen when these peptides were combined (2.4.2.5). When compared to oxyntomodulin, GLP-1 and glucagon appeared to induce less activation of c-fos in the NTS, but when the presence of c-fos in three neuronal populations in the NTS known to be involved in the regulation of feeding was examined, the effects of the three peptides were largely indistinguishable.

To identify whether the glucagon receptor plays any role in the anorectic effects of glucagon or oxyntomodulin, feeding studies using a glucagon receptor antagonist or glucagon receptor knockout mice could be carried out. It would be interesting to examine the effects of these peptides on c-fos expression in the GLP-1R knockout mice, to see whether, as the results of this chapter would suggest, activation of appetite regulating centres is abolished.

The work in this chapter has began to examine neuronal populations in the NTS which may mediate the anorectic effects of the preproglucagon derived peptides, but the majority of the neuronal activation seen does not appear to be accounted for by catecholaminergic or POMC expressing neurons. Further work to identify the activated neurons could be carried out, and may be useful, not only to further our understanding of the mechanism of action of the PPG derived peptides, but perhaps of other anorectic gut hormones.
4 EXPERIMENTAL CHAPTER THREE

The effects of glucagon and oxyntomodulin on glucose homeostasis
4.1 **INTRODUCTION**

4.1.1 **Glucose**

Glucose is a hexose monosaccharide and an important metabolic substrate. The metabolism of glucose begins with glycolysis, which results in the conversion of glucose to pyruvate whilst generating ATP. Pyruvate produced is then further metabolised, under aerobic conditions, via the citric acid cycle and electron transport chain.

In mammals the availability of glucose as a metabolic fuel affects the functioning of numerous physiological processes and is of particular importance to the brain. Levels of glucose in the blood are maintained within a narrow range (between 4 and 7 mmol/l) in both the fasted state and post-prandially. The consequences of failure to maintain glucose levels within this range can be seen in patients with type 1 or type 2 diabetes. Chronic hyperglycemia in diabetics commonly leads to neuropathy (Dyck et al., 1993). High intracellular glucose levels can cause metabolic disturbances in neurons and other cells due to overactivity of the electron transport chain (Vincent et al., 2011). Acute hypoglycaemic episodes in diabetics can result in cognitive dysfunction, seizure and coma and as a result are associated with significant morbidity and mortality (Cryer, 1997; Holstein et al., 2002).

4.1.2 **Glucose homeostasis**

Without regulation fluctuations in glucose would occur over the course of the day. Glucose from digested sugars and carbohydrates is taken up by the intestine post prandially. The uptake and storage of this glucose is crucial to the maintenance of blood glucose levels between meals. Regulatory mechanisms exist at the level of glucose absorption from the intestine, uptake into the tissues, and in glucose storage to prevent and counter any fluctuations.

4.1.2.1 **Glucose sensing**

The regulation of blood glucose relies on the ability to sense changes in circulating glucose concentration. Glucose sensing is involved both directly and indirectly in the release of glucoregulatory hormones. There is no one glucose sensor in the body, instead glucose
concentrations are measured at numerous sites, all of which can respond in different ways to make any necessary alterations. To prevent large variations in blood glucose, glucose sensing begins at the pre-absorptive stage in the intestine and continues through to monitoring of portal glucose as well as circulating glucose and concentrations in the brain. The pancreas is thought to be an important centre for sensing of circulating glucose.

4.1.2.1.1 Pancreatic glucose sensing

The release of insulin and glucagon is directly regulated by glucose levels in the pancreas. Glucose sensing in the alpha and beta cells of the pancreas is thought to be reliant on the presence of glucokinase and of a subset of potassium channels, the K\textsubscript{ATP} channels. At rest, during fasting conditions, the β-cell membrane has a resting potential of around -60mV. When glucose concentrations in the islet rise, glucose is taken up into the β-cell and fed into the glycolytic pathway, of which glucokinase catalyses the first stage. The ATP generated leads to the closure of K\textsubscript{ATP} channels and a resultant depolarisation of the β-cell membrane to around -40mV. Voltage gated Na\textsuperscript{+} and Ca\textsuperscript{2+} channels begin to open, leading to further depolarisation of the membrane. Once the membrane potential reaches around -20mV high voltage Ca\textsuperscript{2+} channels open, leading to a large influx of calcium which triggers exocytosis of insulin from the β-cell (Figure 4.1).
Figure 4.1: Glucose sensing in the pancreatic β-cell. 1. glucose enters the β-cell and is metabolised by the glycolytic pathway. 2. ATP concentration in the cell increases 3. ATP dependant $K_{ATP}$ channels close 4. Build up of $K^+$ ions and resultant resultant depolarisation of the β-cell membrane to -40mV. 5. Voltage gated $Na^+$ and $Ca^{2+}$ channels open 6 influx of calcium triggers exocytosis of insulin from the β-cell.

Pancreatic α-cells possess similar glucose sensing machinery to β-cell, but they need to be able to respond to low levels of glucose, as they are responsible for the production and release of glucagon. The mechanism of glucagon release in response to low glucose levels is not clearly understood. Like β-cells, the α-cell expresses large numbers of $K_{ATP}$ channels (Bokvist et al., 1999). However in the α-cell the $K_{ATP}$ channels are mostly closed at low glucose concentrations, meaning low glucose concentrations cause depolarisation of the α-cell, action potential firing and glucagon release (Gopel et al., 2000). $K_{ATP}$ channel activation by high concentrations of diazoxide (a potassium channel activator) prevents glucagon secretion (MacDonald et al., 2007). This is thought to occur due to a rapid depolarisation of the membrane without subsequent repolarisation which is required to
reactivate voltage gated ion channels, thus although there is a transient release of glucagon, there is a sustained inhibition of release (Rorsman et al., 2008). The reason for the difference in sensitivity of \( K_{ATP} \) channel opening in response to extracellular glucose concentrations between \( \alpha \)-cells and \( \beta \)-cells is not known.

### 4.1.2.1.2 Intestinal glucose sensing

Glucose sensing in the intestines is important for preventing large fluctuations in blood glucose after meal ingestion. An increase in glucose levels in the intestine leads to the release of the incretins GLP-1 and GIP. Glucose is thought to be detected both by sweet taste receptors expressed on the enteroendocrine cells of the small intestine (Dyer et al., 2005; Jang et al., 2007; Margolskee et al., 2007), and via depolarisation induced by the uptake of glucose by the sodium glucose cotransporter SGLT1.

### 4.1.2.1.3 Hepatic-portal glucose sensing

Blood leaving the intestines enters the portal circulation and is carried to the liver, thus glucose sensing in this system can determine the potential for a rise in glucose before it has entered the systemic circulation. Fibres in the hepatic branch of the vagus nerve are sensitive to glucose administration into the portal vein (Niijima, 1982) and low concentrations of glucose in the portal vein stimulate insulin release (Fukaya et al., 2007).

### 4.1.2.1.4 Glucose sensing in the brain

As an organ with a particular reliance on glucose as a fuel, it is unsurprising that glucose sensing occurs in the brain. Glucose sensitive neurons can be either excited or inhibited by rises or falls in local glucose levels. In the hypothalamus glucose sensitive neurons have been found in the ARC, VMH, PVN and LH (Dunn-Meynell et al., 1998; Silver and Erecinska, 1998). In the ARC, 40% of glucose inhibited neurons express NPY (Mountjoy et al., 1994) whilst POMC neurons are excited by glucose (Ibrahim et al., 2003). In the LH MCH expressing neurons are excited by glucose whereas firing in the orexin expressing neurons is inhibited by glucose (Burdakov et al., 2005). The brainstem is also
thought to be involved in central glucose sensing and glucose homeostasis. Glucose sensitive neurons are found in the brainstem, particularly in the caudal part of the NTS (Adachi et al., 1984; Mizuno and Oomura, 1984) and many of these are thought to be catecholaminergic (Yettefti et al., 1997).

Neurons that are excited by high concentrations of glucose are thought to sense glucose levels by a similar mechanism to the pancreatic β-cell. K_{ATP} channel subunits are expressed in glucose excited ARC POMC, LH MCH neurons and in glucose excited neurons in the dorsovagal complex (Ibrahim et al., 2003; Kong et al., 2010; Balfour et al., 2006) and selective genetic deletion or pharmacological blockade of these channels affects their ability to respond to glucose (Parton et al., 2007; Kong et al., 2010; Balfour et al., 2006). In glucose inhibited neurons it is thought that the production of ATP by glucose metabolism might increase the activity of the 3Na^+2K^+ ATPase inhibiting cellular depolarisation (Silver and Erecinska, 1998). Reactive oxygen species (ROS) and 5' adenosine monophosphate-activated protein kinase (AMPK) have also been implicated in neuronal glucose sensing (Leloup et al., 2006; Claret et al., 2007).

### 4.1.2.2 Glucose stores

As important as the ability to sense changes in blood glucose is the ability for excess glucose to be stored for use in times of fasting. Glucose itself is not stored in large quantities within tissues and instead is stored as an osmotically neutral polymer: glycogen. Glycogen is formed of α-1, 4-glycosidic bonds between glucose residues with α-1, 6-glycosidic bonds creating branch points. The major glycogen stores are in the skeletal muscle and liver, but the kidney, heart, brain and adipose tissue can also synthesise glycogen.

### 4.1.2.3 Endocrine Regulation of Blood Glucose

Glucoregulatory hormones are released in response to signals from glucose sensitive tissues. In mammals insulin and glucagon are the major regulators of blood glucose.
4.1.2.3.1 Insulin

Insulin is a peptide hormone composed of an A-chain and a B-chain connected by two disulphide bonds, and in humans is 51 amino acids in total. The insulin gene is present in one copy in humans but two copies in rats and mice (Lomedico et al., 1979) and is expressed only in the pancreatic islets (Su et al., 2004). Transcription of the insulin gene leads to the production of preproinsulin mRNA, which is translated into the insulin precursor preproinsulin. Preproinsulin consists of an N-terminal signal peptide, followed by the B-chain which is connected to the A-chain by a linking peptide known as C-peptide. The removal of the signal peptide produces proinsulin in which the A and B-chains are joined by disulphide bonds, but also by the intervening C-peptide sequence. C-peptide is cleaved from the A and B-chains by endopeptidases once proinsulin has been packaged into immature granules. These granules then mature and insulin is stored within them until release.

Insulin release is considered to occur in two phases. The first phase of insulin secretion includes what is known as the cephalic phase, which occurs prior to nutrient absorption or any change in blood glucose and prevents large oscillations in blood glucose post prandially. The remainder of first phase insulin secretion occurs in response to elevated blood glucose. First phase insulin secretion is the release of what is known as the readily releasable pool, thought to be pre-docked insulin containing granules (Daniel et al., 1999). This occurs within 10 minutes of exposure to sufficient concentrations of glucose in vivo. The second phase which follows this involves the mobilisation, docking and release of reserve granules reaches a plateau at around 30 minutes after stimulation.

4.1.2.3.1.1 Endocrine regulation of insulin secretion

Although glucose is the main regulator of insulin secretion, the magnitude of glucose stimulated insulin release is affected by the presence of several hormones. Glucagon, GLP-1 and GIP can act directly on receptors on β-cells to increase cAMP and thus activate protein kinase A (PKA) (Moens et al., 1996). PKC activation also influences glucose stimulated insulin release. Activation of PKA or PKC
reduces the threshold intracellular calcium concentration required for insulin release (Hughes et al., 1989).

4.1.2.3.1.2 Central regulation of insulin secretion

Regulation of insulin secretion by the nervous system occurs both during the cephalic phase and post-absorptive phases. The pancreatic islets receive innervation from both the sympathetic and parasympathetic branches of the autonomic nervous system (Esterhuizen et al., 1968; Coupland, 1958) Activity of the parasympathetic branch is associated with insulin release in response to circulating glucose levels and also in the incretin effect of GLP-1 (Nagase et al., 1993; Balkan and Li, 2000).

Parasympathetic fibres release acetylcholine (ACh) alongside a number of neuropeptides including vasoactive intestinal peptide (VIP), gastrin releasing peptide (GRP), and pituitary adenylate cyclase-activating poly-peptide (PACAP). Parasympathetic activity stimulates insulin release (Ahren et al., 1986; Brunicardi et al., 1995) and this is thought to be a direct action of ACh on beta cell muscarinic receptors. ACh binding to muscarinic receptors stimulates phospholipase C (PLC), increasing Inositol triphosphate levels and consequently releasing Ca\(^{2+}\) from intracellular stores. VIP, GRP and PACAP also increase glucose-stimulated insulin release (Fridolf et al., 1992; Ahren and Lundquist, 1981; Knuhtsen et al., 1987) by binding to adenylate cyclase-activating G-protein coupled receptors.

Conversely, sympathetic stimulation of the pancreas inhibits basal and glucose stimulated insulin secretion (Bloom and Edwards, 1984; Ahren et al., 1987). This is thought to be an action of noradrenaline on \(\alpha_2\)-adrenoreceptors (Porte, Jr. and Williams, 1966; Skoglund et al., 1988). Activation of \(\alpha_2\)-adrenoreceptors leads to opening of \(K_{ATP}\) channels and thus hyperpolarisation of beta cells (Nilsson et al., 1988).

4.1.2.3.1.3 Insulin Receptor

The insulin receptor belongs to a family of receptors all containing a region with tyrosine kinase activity (Ebina et al., 1985). The insulin receptor is widely expressed, with highest expression in the
kidney, adrenal cortex, pituitary, heart, liver, skeletal muscle, prefrontal cortex and hypothalamus (Su et al., 2004). Insulin binding causes autophosphorylation of several tyrosine residues on the intracellular domain of the receptor (Kasuga et al., 1982) and phosphorylation of insulin receptor substrates (IRS). Phosphorylated IRS bind to numerous signalling molecules, including PI3K, and stimulate the activation of several major intracellular signalling cascades (Saltiel and Kahn, 2001).

4.1.2.3.1.4 Insulin action

Insulin coordinates the response to hyperglycemia; increasing glucose disposal by stimulating glucose uptake, glycogen synthesis and glucose utilisation, whilst reducing glucose output by inhibition of glycogenolysis and gluconeogenesis. Regulation of glucose uptake by insulin is mainly via GLUT4. In the absence of insulin GLUT4 is mostly localised to endosomal, golgi or vesicular membranes within the cell. Insulin receptor activation triggers the translocation of GLUT4 to the cell membrane (Klip et al., 1987). GLUT4 is rapidly endocytosed such that, should insulin levels fall in response to reduced blood glucose, GLUT4 mediated glucose uptake will also fall (Leto and Saltiel, 2012). Once glucose has been taken up by the tissues, insulin increases glucose storage in the liver and skeletal muscle by stimulation of glycogen synthase activity (Parker et al., 1983).

Regulation of glucose output by insulin occurs via inhibition of gluconeogenesis and glycogenolysis. In addition to reducing glucose uptake and therefore substrate availability, insulin activates hepatic insulin receptors to regulate the activity of several enzymes involved in the glycolytic/gluconeogenic pathway (Pilkis and Granner, 1992; Michael et al., 2000). Insulin also negatively regulates the transcription of phosphoenolpyruvate carboxykinase (PEPCK), the rate limiting enzyme in gluconeogenesis (Sutherland et al., 1996).
4.1.2.3.2 Glucagon

Glucagon is released when blood glucose levels fall (section 4.1.2.1.1) and acts to increase glucose concentration by stimulating glycogenolysis and gluconeogenesis (summary in figure 4). Like insulin, glucagon can be released by a direct effect of glucose concentration in the pancreatic islets and also by nervous stimulation (Olsen et al., 2005). The nervous stimulation of glucagon release is thought to be particularly important, with 75-80% of hypoglycaemia induced glucagon release dependent on autonomic nervous input (Havel and Ahren, 1997).

4.1.2.3.2.1 Hyperglycemic effects of glucagon

Elevation of blood glucose in response to glucagon occurs by three complementary actions. Glucagon prevents further synthesis of glycogen by inhibiting the action of glycogen synthase (Curnow et al., 1975). It simultaneously promotes glycogen breakdown by stimulation of glycogen phosphorylase (Levine, 1965) and glucose-6-phosphatase (Band and Jones, 1980). As well as triggering glucose release from stored glycogen, glucagon also stimulates gluconeogenesis by increasing the expression of PEPCK (Jiang and Zhang, 2003).
Figure 4.2: Glucagon receptor signalling to increase cellular glucose output. AC, Adenylate cyclase; ATP, Adenosine triphosphate; cAMP, cyclic Adenosine monophosphate; GP, Glycogen phosphorylase; GPK, Glycogen phosphorylase kinase; GS, Glycogen synthase; G-6-Pase, glucose-6-phosphatase; IP₃,
4.1.2.3.2 Insulinotropic effect of glucagon

The release of insulin by glucagon is a well documented, but paradoxical effect of this hyperglycemic hormone. Glucagon receptors are expressed on β-cells (Goldfine et al., 1972) and glucagon stimulates insulin release, although less potently than GLP-1 (Weir et al., 1989). The difference in potency of GLP-1 and glucagon to stimulate insulin release lead to the suggestion that glucagon stimulates insulin release by crossreaction with the GLP-1R. However it was shown that the GLP-1R antagonist EX 9-39 did not block glucagon-induced insulin release (Kawai et al., 1995; Gromada et al., 1997b). There is however some evidence for participation of the GLP-1R in glucagon-stimulated insulin release (Marie et al., 1996) and some have suggested that both receptors may be involved (Moens et al., 1998). Currently there is no data regarding the relevance of the glucagon and GLP-1Rs to the in vivo stimulation of insulin release by glucagon.

4.1.2.4 Oxyntomodulin and glucose homeostasis

Despite being an agonist at both the glucagon and GLP-1Rs, the GLP-1R mediated effects of oxyntomodulin on blood glucose appear to dominate. Acute administration of oxyntomodulin improves glucose tolerance in mice (Maida et al., 2008; Parlevliet et al., 2008). This effect is absent in GLP-1R knockout mice (Maida et al., 2008) suggesting the improvement is due to binding at the GLP-1R. It is not clear whether oxyntomodulin binding at the glucagon receptor is relevant to its effects on glucose and insulin release in vivo. In cows, a glucagon receptor antagonist reduced the insulin release induced by oxyntomodulin, although to a lesser extent than a GLP-1R antagonist, suggesting the glucagon receptor has a role in oxyntomodulin-induced insulin release (ThanThan et al., 2012). In this chapter the effects of oxyntomodulin on blood glucose and the role of the GLP-1R in mediating these effects will be investigated in murine models.
4.1.3 Chapter Summary and Aims

Whilst acute administration of oxyntomodulin is known to improve glucose tolerance via its action at the GLP-1R, little is known about whether oxyntomodulin’s activity at the glucagon receptor has any effects on blood glucose in vivo. The experiments contained within this chapter aimed to elucidate the contribution of GLP-1 and glucagon receptor activation to the effects of oxyntomodulin on blood glucose and insulin release in mice. However, in the course of these experiments it was noted that the effects of glucagon and oxyntomodulin on blood glucose are not as simple as the literature might suggest, and the mechanism behind these discrepancies was investigated. EX 9-39, a GLP-1R antagonist, was used as a tool to investigate the effects of GLP-1R activation by oxyntomodulin and glucagon on blood glucose in mice, and it too had unexpected effects on glucose homeostasis, which are also investigated in the experiments within this chapter.
4.2 MATERIALS AND METHODS

4.2.1 Measurement of the glucose and insulin response to peripherally administered peptides

4.2.1.1 Animals

All animal procedures undertaken were approved by the British Home Office under the UK Animal (Scientific Procedures) Act 1986 (Project license 70/6402). Male C57Bl/6 mice (Harlan, UK) or GLP-1R knockout mice were singly housed in individually ventilated cages under controlled temperature (21-23°C) and a 12:12 hour light-dark cycle (lights on 0700). Animals had ad libitum access to food (RM1 diet, Special Diet Services Ltd, Witham, UK) and water unless otherwise stated.

4.2.1.2 Study procedure

Studies were designed to measure and compare the acute effects of glucagon and oxyntomodulin on blood glucose and insulin release. To allow enough time to take all the required measurements at the specified timepoints these studies were carried out over two or three day periods, with an equal number of animals from each group being tested on each day. In all studies mice were in the fed state so as to be able to observe any glycogenolytic effects on blood glucose of these hormones. All studies were carried out in the early light phase when cellular glycogen stores would be expected to be high.

Mice were acclimatised to the study procedure by daily handling and sham injections for seven days preceding the first study. Tail tipping and blood glucose measurements were carried out on two occasions prior to the study. Mice were randomised into groups by body weight.

The studies began at 0900 with blood samples for baseline glucose and insulin measurements taken. Food was removed after the baseline measurement and returned at the end of the test. This was to prevent any effects of the hormones on appetite from influencing the glucose and insulin results. An outline of the study procedure is shown in Figure 4.3. A small cut was made to the tail tip using a
scalpel and a drop of blood used to measure baseline blood glucose using a glucometer t=−30 (CONTOUR meter and test strips, Bayer, Berkshire, UK). Baseline blood glucose was measured 30 minutes prior to injection to reduce the impact of stress induced by the cut on subsequent measurements. Approximately 20 μl of blood for measurement of baseline insulin was collected in Lithium-Heparin microvettces (Starstedt, Leicester, UK) and kept on ice. Mice were returned to their home cages between all measurements. At t=0 mice were injected subcutaneously with either saline or a test peptide at the doses specified. Glucose was measured at t= 5, 15 and 35. A blood sample for measurement of insulin was taken at t=15. Mice were re-fed at the end of the study.

Figure 4.3: Outline of in vivo study procedure when investigating the effects of glucagon or oxyntomodulin on blood glucose.

4.2.1.3 Studies in GLP-1R knockout mice

See section 3.2.1.1 for a description of the origins and genotyping of the GLP-1R KO mice. In studies using GLP-1R KO mice a crossover design was used such that each mouse received all treatments on separate days, with a recovery period in between. Equal numbers of animals received each treatment on any given day. Insulin samples were prepared, stored and assayed as described in section 2.3.2.4.3.1.
4.2.2 Measurement of liver glycogen

Liver glycogen was measured to determine whether both low and high doses of glucagon were capable of stimulating glycogenolysis.

Mice were acclimatised to handling and subcutaneous saline injections daily for seven days prior to the study, and randomised into treatment groups by body weight. Mice were fed ad libitum before subcutaneous injection with either saline or a dose of a specified peptide at 0930 hours, and returned to their home cage at which point their food was removed. After 15 minutes mice were killed by decapitation and their livers were rapidly dissected and snap frozen under liquid nitrogen. Samples of liver were stored at -20°C for up to a week prior to being assayed for glycogen content.

4.2.2.1 Sample preparation

Liver samples were kept on ice at all times during processing. Samples of liver were weighed (weighing between 10 and 20 mg) and added to a 2ml Eppendorf containing 200 μl of ice cold distilled water per 10 mg of weight. Samples were then homogenised for 2 minutes at 20Hz in pre-chilled blocks using stainless steel beads (5 mm diameter, Qiagen, UK) in a Qiagen Tissuelyser II (Qiagen, UK). Homogenised samples were then boiled for 5 minutes to inactivate enzymes before being centrifuged at 13 000 rpm for 5 minutes.

4.2.2.2 Glycogen Assay

The supernatants were assayed for glycogen content using the Abcam Glycogen Assay Kit according to the manufacturer’s instructions (Abcam, Cambridge, UK). This kit uses hydrolysis of glycogen to glucose by a glucoamylase enzyme. Glucose is then oxidised to D-glucono-δ-lactone in a reaction which produces H₂O₂. The H₂O₂ content of the sample is then measured using an OxiRed probe to generate colour. OxiRed reacts with H₂O₂ to produce a coloured product which absorbs maximally at 570 nm. Because background glucose will be present in the liver samples, each sample is assayed with and without the hydrolysis enzyme. Without the addition of the hydrolysis enzyme glycogen will not be broken down to glucose and thus only glucose already present in the tissue will be
measured. This background is then subtracted from the reading from the same sample with hydrolysis enzyme present to give a measurement of glycogen content. Glycogen standards are used to plot a standard curve which, using a linear curve fit as recommended by the manufacturers, can be used to generate values for unknown samples.

4.2.3 Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM) for all studies. The unpaired two tailed T Test was used for comparisons between two groups and one-way analysis of variance (ANOVA) was used for comparisons between three or more independent groups at individual time points. A post hoc Dunnett’s test was used to compare each group to a saline control in dose response studies. Analyses were performed using Prism version 4.03 software (Graphpad Software, San Diego, CA, USA). In cross-over studies, comparing knockout to wild type animals, data were analysed using a mixed-model ANOVA with genotype as the between-subjects variable and treatment as the within-subjects variable followed by Holm-Sidak’s multiple comparison test, which compared all the treatments for animals of the same genotype (Prism version 6.0, Graphpad Software). In all cases the threshold for statistical significance was set at p<0.05.
4.3 RESULTS

4.3.1 The effects of oxyntomodulin on glucose and insulin release in wild type mice

Baseline fed state glucose values did not differ between groups (Figure 4.4 B). At five minutes after injection there were no significant changes in blood glucose in OXM treated animals compared to saline controls (Figure 4.4 C). Fifteen minutes after injection doses of OXM below 1000 nmol/kg appeared to reduce blood glucose, reaching statistical significance in the 30 and 100 nmol/kg groups (Figure 4.4 D), however the 3000 nmol/kg dose had no effect on blood glucose at this timepoint. At the 35 minute timepoint OXM caused a reduction in blood glucose compared to saline controls at doses of 100 nmol/kg and above (Figure 4.4 E).

There were no differences in plasma insulin levels between groups at baseline. All doses of oxyntomodulin appeared to increase insulin levels compared to saline controls 15 minutes after injection, reaching statistical significance at doses of 300 nmol/kg and greater (Figure 4.5).
Figure 4.4: The effect of a range of doses of oxyntomodulin on blood glucose in wild type fed mice. Blood glucose before (-30 minutes) and after (5, 15 and 35 minutes) injection of saline or oxyntomodulin at t=0. injected in the early light phase (0930) s/c and food removed from -30 minutes onwards. N=5-7 *p<0.05, ** p<0.01.
Figure 4.5: The effect of a range of doses of oxyntomodulin on plasma insulin in fed mice. Blood samples taken before (-30 minutes) and after (15 minutes) injection of saline or oxyntomodulin at t=0. Injected in the early light phase (0930) s/c and food removed from -30 minutes onwards. A: baseline plasma insulin (-30 minutes) B: Plasma insulin 15 minutes after injection. N=5-7 *p<0.05, ** p<0.01, *** p<0.001.
4.3.2 The effects of a range of doses of glucagon on glucose and insulin release in wild type mice

For comparison with the effects of oxyntomodulin, a weak glucagon receptor agonist in vitro, the response of ad libitum fed mice to a range of doses of glucagon was also assessed.

Baseline fed state glucose values did not differ between groups (Figure 4.6 B). The 30 nmol/kg dose of glucagon increased blood glucose significantly compared to saline controls at 5, 15 and 35 minutes after injection (Figure 4.6 C,D,E). No change in blood glucose was seen in response to doses of glucagon above 30 nmol/kg.

Plasma insulin levels were not significantly different between groups at baseline. At 15 minutes after injection glucagon caused an increase in plasma insulin, reaching statistical significance in the groups receiving glucagon at 100, 300 and 3000 nmol/kg (Figure 4.7).
Figure 4.6: The effect of a range of doses of glucagon on blood glucose in fed mice. Blood glucose before (-30 minutes) and after (5, 15 and 35 minutes) injection of saline or glucagon at t=0. injected in the early light phase (0930) s/c and food removed from -30 minutes onwards. N=6 *p<0.05, ** p<0.01, ***p<0.001.
Figure 4.7: The effect of a range of doses of glucagon on plasma insulin in fed mice. Blood samples taken before (-30 minutes) and after (15 minutes) injection of saline or glucagon at t=0. Injected in the early light phase (0930) s/c and food removed from -30 minutes onwards. A: baseline plasma insulin (-30 minutes) B: Plasma insulin 15 minutes after injection. N=5-7 *p<0.05, ** p<0.01, *** p<0.001.
4.3.3 The effects of a range of low doses of glucagon on glucose and insulin release in wild type mice

Study 4.3.2 was repeated at lower doses of glucagon to determine whether a hyperglycemic effect would be present.

Baseline fed state glucose values did not differ significantly between groups (Figure 4.8 B). At five minutes after injection there were no significant changes in blood glucose in glucagon treated animals compared to saline controls (Figure 4.8 C). Fifteen minutes after injection there was a significant elevation in blood glucose in the group receiving 10 nmol/kg of glucagon compared to saline controls (Figure 4.8 D). There was no significant difference in blood glucose in glucagon treated animals compared to saline controls at 35 minutes after injection (Figure 4.8 E).

Plasma insulin levels were slightly different at baseline between different groups in this study, with the group later to receive 30 nmol/kg glucagon having statistically higher baseline insulin levels than the group which later received saline injections (Figure 4.9 A). Fifteen minutes after injection the 100 nmol/kg glucagon group had significantly higher plasma insulin than saline controls (Figure 4.9 B).
Figure 4.8: The effect of a range of doses of glucagon on blood glucose in fed mice. Blood glucose before (-30 minutes) and after (5, 15 and 35 minutes) injection of saline or glucagon at t=0, injected in the early light phase (0930) s/c and food removed from -30 minutes onwards. N=6 *p<0.05, ** p<0.01, ***p<0.001.
Figure 4.9: The effect of a range of doses of glucagon on plasma insulin in fed mice. Blood samples taken before (-30 minutes) and after (15 minutes) injection of saline or glucagon at t=0. Injected in the early light phase (0930) s/c and food removed from -30 minutes onwards. A: baseline plasma insulin (-30 minutes) B: Plasma insulin 15 minutes after injection. N=5-7 *p<0.05, ** p<0.01, *** p<0.001.
4.3.4 Effects of glucagon and oxyntomodulin on blood glucose and insulin

Figure 4.10 shows a compilation of the results of studies 4.3.2 and 4.3.3 for comparison of the effects of the entire range of glucagon doses tested on blood glucose and plasma insulin. Figure 4.11 shows the effects of the range of oxyntomodulin doses tested for comparison.

Figure 4.10: The effect of a range of doses of glucagon on plasma glucose and insulin in fed mice. Compiled from data from studies (4.3.2 and 4.3.3).

Figure 4.11: The effect of a range of doses of oxyntomodulin on plasma glucose and insulin in fed mice. Data from studies 4.3.1.
4.3.5 The effect of a high dose of glucagon on glucose release shortly after injection in wild type mice

Due to the unexpected lack of hyperglycemic effect after high (100 nmol/kg and greater) doses of glucagon, blood glucose was measured in response to 3000 nmol/kg glucagon at timepoints 1 and 2 minutes after injection to test the hypothesis that high doses of glucagon have a more rapid hyperglycemic effect, which is counteregulated by insulin release such that no change in blood glucose is evident by 5 minutes after administration.

Baseline fed state glucose values did not differ significantly between groups (Figure 4.12). There was no difference in blood glucose between saline and glucagon treated animals at any of the timepoints tested (Figure 4.12).
Figure 4.12: The effect of high dose glucagon blood glucose in fed mice. Blood glucose before (-30 minutes) and after (1, 2, 15 and 35 minutes) injection of saline or glucagon at t=0. Injected in the early light phase (0930) s/c and food removed from -30 minutes onwards. N=6.
4.3.6 The effect of low and high dose glucagon on liver glycogen levels

Liver glycogen content was assessed to compare the effects of low and high doses of glucagon on glucose mobilisation from liver glycogen stores. Neither treatment significantly altered liver glycogen levels compared to saline controls (Figure 4.13).

Figure 4.13: The effect of high and low dose glucagon on liver glycogen content in fed mice. Fed mice injected in the early light phase (0930) s/c and food removed, killed 15 minutes later and liver samples removed and snap frozen for later processing. Glycogen values given as μg of glycogen per mg of wet liver. N=7-9.
4.3.7 The effect of high and low dose glucagon on blood glucose and insulin release in wild type and GLP-1R knockout mice

In order to test the hypothesis that the release of insulin by high doses of glucagon was due in some part to cross-reaction with the GLP-1R glucagon was administered to GLP-1R knockout mice and wild type littermate controls. This study also aimed to determine whether GLP-1R mediated actions of high doses of glucagon might explain the lack of hyperglycemic effect.

Two doses of glucagon, low (10 nmol/kg) and high (100 nmol/kg) were administered to GLP-1R knockout and wild type littermate controls. The 10 nmol/kg dose was chosen as the dose which caused the greatest elevation in blood glucose in study 4.3.3. 100 nmol/kg was chosen as a dose which reliably did not cause an elevation in glucose, but did cause significant insulin release. A higher dose was not selected as the aim of these studies was to determine whether the GLP-1R contributed to the insulinotropic effect of glucagon. If there is an overabundance of peptide the absence of the GLP-1R might not have any effect on the insulin released, regardless of whether glucagon can act partly via this receptor, because insulin release may be at a maximum.

A two way ANOVA found there was a statistically significant effect of genotype on blood glucose at baseline and at 35 minutes post injection. A significant main effect of treatment on blood glucose was seen at baseline, and at 15 and 35 minutes post injection. There was no significant genotype*treatment interaction effect at any timepoint.

Post hoc analysis showed 15 minutes after 10 nmol/kg glucagon administration blood glucose was significantly elevated compared to saline controls in both wild type and GLP-1R knockout mice (Figure 4.14 B). Fifteen minutes after 100 nmol/kg glucagon administration blood glucose was significantly elevated compared to saline controls in GLP-1R knockout mice, but not in wild type mice (Figure 4.14 D). This was reflected by a statistically significant difference in blood glucose between wild type and GLP-1R knockout mice receiving 100 nmol/kg glucagon 15 minutes after injection. Post
hoc tests at 35 minutes after injection showed no significant effects of either 10 or 100 nmol/kg of glucagon in wild type or GLP-1R knockout mice (Figure 4.14 E)

A two way ANOVA found no statistically significant main effect of treatment or genotype on plasma insulin at baseline. Fifteen minutes after injection there was a significant main effect of treatment on plasma insulin. There was no significant treatment*genotype interaction at either time.

Post hoc analysis showed significantly greater plasma insulin levels 15 minutes after 100 nmol/kg glucagon compared to saline injection in both wild type and GLP-1R knockout mice, but 10 nmol/kg glucagon increased insulin levels only in GLP-1R knockout mice (Figure 4.15 B). Post hoc analysis showed no statistically significant effect of genotype on the response to any of the treatments.
Figure 4.14: The effect of high and low dose glucagon on blood glucose in wild type and GLP-1R knockout mice. Blood glucose before (-30 minutes) and after (5, 15 and 35 minutes) injection of saline or glucagon (10 or 100 nmol/kg) at t=0. Injected in the early light phase (0930) s/c and food removed from -30 minutes onwards. N=9. *p<0.05, ** p<0.01, *** p<0.001., with *comparing treatments to saline, $ comparing 100nmol/kg to 10nmol/kg glucagon and # comparing the effect of genotype on mice receiving the same treatment.
Figure 4.15: The effect of high and low dose glucagon on plasma insulin in wild type and GLP-1R knockout mice. Blood samples taken before (-30 minutes) and after (15 minutes) injection of saline or glucagon (30 or 3000 nmol/kg) at t=0. Injected in the early light phase (0930) s/c and food removed from -30 minutes onwards. A: baseline plasma insulin (-30 minutes) B: Plasma insulin 15 minutes after injection. N=9 *p<0.05, ** p<0.01, *** p<0.001, with *comparing treatments to saline treatment, $ comparing 100 nmol/kg to 10 nmol/kg glucagon and # comparing the effect of genotype on mice receiving the same treatment.
4.3.8 The effect of high and low dose oxyntomodulin on blood glucose and insulin release in wild type and GLP-1R knockout mice

In order to investigate the effect of oxyntomodulin binding to the GLP-1R on insulin release and blood glucose, oxyntomodulin was administered to GLP-1R knockout mice. Two doses of oxyntomodulin, low (30 nmol/kg) and high (3000 nmol/kg) were administered to GLP-1R knockout and wild type littermate controls. The 30 nmol/kg dose was chosen as it caused a reliable decrease in blood glucose in study 4.3.1. 3000 nmol/kg was chosen as the only dose tested not to cause a decrease in blood glucose 15 minutes after injection. This study aimed to determine whether the GLP-1R is required for the reduction in blood glucose seen in response to 30 nmol/kg of glucagon, and whether either dose of oxyntomodulin was capable of increasing blood glucose in the GLP-1R knockout mice.

There was a statistically significant main effect of genotype on blood glucose at baseline and at 35 minutes post injection. A significant main effect of treatment was seen 5 minutes post injection and there was a significant treatment*genotype interaction effect at 15 and 35 minutes post injection.

Post hoc analysis showed a significantly significant rise in blood glucose in wild type mice 5 minutes after injection of 30 nmol/kg oxyntomodulin (Figure 4.16 C). Fifteen minutes post injection neither treatment caused a statistically significant alteration in blood glucose compared to saline controls, but the 30 nmol/kg dose of oxyntomodulin appeared to reduce blood glucose in wild type mice (p=0.057) and the 3000 nmol/kg dose of oxyntomodulin appeared to increase blood glucose compared to the 30 nmol/kg dose in wild type mice (p=0.057)(Figure 4.16 D). Fifteen minutes after injection blood glucose was significantly higher in all groups of GLP-1R knockout mice compared to wild type mice receiving the same treatment. Post hoc tests showed a significant reduction in blood glucose in wild type mice receiving 30 nmol/kg oxyntomodulin compared to saline controls and a significant difference in blood glucose between GLP-1R knockout and wild type mice receiving 30 or 3000 nmol/kg of oxyntomodulin 35 minutes post injection (Figure 4.16 E).
There was no statistically significant main effect of treatment or genotype on plasma insulin at baseline. Fifteen minutes after injection there was a significant main effect of treatment on blood glucose. There was no significant treatment*genotype interaction at either time.

Post hoc analysis showed significantly greater plasma insulin levels 15 minutes after both 30 and 3000 nmol/kg oxyntomodulin injections in wild type mice with no effect in GLP-1R knockout mice (Figure 4.17 B).
**Figure 4.16:** The effect of high and low dose oxyntomodulin on blood glucose in wild type and GLP-1R knockout mice. Blood glucose before (-30 minutes) and after (5, 15 and 35 minutes) injection of saline or oxyntomodulin (30 or 3000 nmol/kg) at t=0. Injected in the early light phase (0930) s/c and food removed from -30 minutes onwards. N=9. *p<0.05, ** p<0.01, *** p<0.001., with *comparing treatments to saline, $ comparing 100nmol/kg to 10nmol/kg glucagon and # comparing the effect of genotype on mice receiving the same treatment.
Figure 4.17: The effect of high and low dose of oxyntomodulin on plasma insulin in wild type and GLP-1R knockout mice. Blood samples taken before (-30 minutes) and after (15 minutes) injection of saline or oxyntomodulin (30 or 3000nmol/kg) at t=0. Injected in the early light phase (0930) s/c and food removed from -30 minutes onwards. A: baseline plasma insulin (-30 minutes) B: Plasma insulin 15 minutes after injection. N=9  *p<0.05, ** p<0.01, *** p<0.001., with *comparing treatments to saline treatment, $ comparing 100nmol/kg to 10nmol/kg glucagon and # comparing the effect of genotype on mice receiving the same treatment.
4.3.9 The effect of high and low dose glucagon on blood glucose in the presence and absence of EX 9-39

This study aimed to use EX 9-39 as a tool to block the GLP-1R, and thus to functionally replicate studies in GLP-1R knockout mice without the confounding differences in baseline glucose seen between GLP-1R knockout and wild type mice, and without the potential effects of developmental compensation.

There was no statistically significant effect of glucagon treatment at either dose on blood glucose at any of the timepoints tested (Figure 4.18). Blood glucose was significantly higher in mice receiving EX 9-39 compared to saline controls at the 5, 15 and 35 minute timepoints. There was a statistically significant difference in blood glucose between mice receiving 10 nmol/kg glucagon with and without EX 9-39 at the 15 and 35 minute timepoints. There was a statistically significant difference in blood glucose between mice receiving 100 nmol/kg glucagon with and without EX 9-39 at the 5, 15 and 35 minute timepoints.
Figure 4.18: The effect of high and low dose glucagon on blood glucose in mice in the presence and absence of a GLP-1R antagonist. Blood glucose before (-30 minutes) and after (5, 15 and 35 minutes) injection of saline (white bars) or EX-9-39 (25 μmol/kg) (shaded bars) at t=-5 and saline or glucagon (10 or 100 nmol/kg) at t=0. Injected in the early light phase (0930) s/c and food removed from -30 minutes onwards. N=6. p<0.05, ** p<0.01, *** p<0.001.
4.3.10 The effects of a range of doses of EX 9-39 on glucose in fed wild type mice

Study 4.3.9 showed an unexpected hyperglycemic effect of EX 9-39 on blood glucose. The effects of a range of doses of EX 9-39 on blood glucose were investigated to determine whether a dose could be found that would not affect blood glucose, and as such could be used as a GLP-1R antagonist in studies examining the mechanisms of action of glucagon and oxyntomodulin. The timepoints used in this study are five minutes later than those used in the other studies in this chapter because EX 9-39 was given at t=-5 when in combination with other peptides.

Baseline fed state glucose values did not differ between groups (Figure 4.19 B). At ten minutes after injection blood glucose was significantly elevated in the 10 000 nmol/kg EX 9-39 group compared to saline controls (Figure 4.19 C). Twenty minutes after injection all doses of EX 9-39 used in this study increased blood glucose compared to saline controls (Figure 4.19 D). At 40 minutes after injection glucose remained elevated in the animals receiving 1000 nmol/kg EX 9-39 and greater (Figure 4.19 E).
Figure 4.19: The effect of a range of doses of the GLP-1R antagonist EX 9-39 on blood glucose in fed mice. Blood glucose before (-30 minutes) and after (10, 20 and 40 minutes) injection of saline or EX-9-39 at t=0. Injected in the early light phase (0930) s/c and food removed from -30 minutes onwards. N=6. p<0.05, ** p<0.01, *** p<0.001.
4.3.11 The effect of EX 9-39 on blood glucose and insulin release in wild type and GLP-1R knockout mice

To determine whether the effects of EX 9-39 on blood glucose in experiment 4.3.9 were dependant on the GLP-1R, EX 9-39 was administered to GLP-1R knockout mice and their wild type littermates.

A two way ANOVA found a significant main effect of genotype at ten minutes post injection. There was a significant main effect of treatment 10, 20 and 40 minutes post injection. There was no significant treatment*genotype interaction effect at any timepoint measured.

Post hoc analysis showed blood glucose to be significantly higher in EX 9-39 treated mice compared to saline controls 10, 20 and 40 minutes after injection in both the wild type and GLP-1R knockout groups (Figure 4.20). There was no significant difference between blood glucose in the wild type and GLP-1R knockout mice at any timepoint measured.

There was no statistically significant main effect of treatment or genotype, nor a significant treatment*genotype interaction, on plasma insulin at baseline or 15 minutes after injection. Post hoc analysis showed no significant difference between insulin levels at either timepoint (Figure 4.21).
Figure 4.20: The effect of the GLP-1R antagonist EX 9-39 on blood glucose in wild type and GLP-1R knockout mice. Blood glucose before (-30 minutes) and after (10, 20 and 40 minutes) injection of saline or EX-9-39 at t=0. Injected in the early light phase (0930) s/c and food removed from -30 minutes onwards. N=4-5. p<0.05, ** p<0.01, *** p<0.001.
Figure 4.21: The effect of the GLP-1R antagonist EX 9-39 on plasma insulin in wild type and GLP-1R knockout mice. Blood samples taken before (-30 minutes) and after (20 minutes) injection of saline or EX 9-39 at t=0. Injected in the early light phase (0930) s/c and food removed from -30 minutes onwards. A: baseline plasma insulin (-30 minutes) B: Plasma insulin 15 minutes after injection. N=5-7 *p<0.05, ** p<0.01, *** p<0.001.
4.4 DISCUSSION

The experiments within this chapter aimed to investigate the contribution of GLP-1R activation by oxyntomodulin to its effects on blood glucose and insulin release and whether, in the absence of GLP-1R activation, oxyntomodulin was capable of inducing a hyperglycemic effect. It was found that in wild type mice oxyntomodulin reduced blood glucose at most doses tested, but at a high dose this effect was delayed. All doses of oxyntomodulin appeared to increase insulin levels in wild type mice. In GLP-1R knockout mice however the insulinotropic effect of oxyntomodulin was abolished, moreover there was no hypoglycaemic effect. The effects of glucagon on blood glucose were dose dependant such that high doses did not have a hyperglycemic effect but did release insulin. In GLP-1R knockout mice however, glucagon was hyperglycemic at higher doses suggesting the presence of the GLP-1R is involved in the unexpected lack of hyperglycemic effect at high doses in wild type mice. EX 9-39 was used to block the GLP-1R pharmacologically, but was found to have its own hyperglycemic effect.

4.4.1 Oxyntomodulin

4.4.1.1 The effects of oxyntomodulin on blood glucose and plasma insulin in wild type mice

As expected, acute administration of oxyntomodulin increased plasma insulin in wild type mice in a dose-dependent manner. The dose response relationship for the effect of oxyntomodulin on blood glucose was more complicated. Despite increasing plasma insulin to a lesser extent than the higher doses of oxyntomodulin, the lowest two doses (30 and 100 nmol/kg) appeared to cause a greater reduction in blood glucose 15 minutes after administration. At this timepoint the highest dose of oxyntomodulin (3000 nmol/kg) had no effect on blood glucose compared to the saline control, despite causing the largest increase in plasma insulin. This may suggest that the hyperglycemic effect of glucagon receptor activation becomes relevant at higher doses, and thus counteracts the insulinotropic effects.
4.4.1.2 The effect of oxyntomodulin on blood glucose and insulin release in GLP-1R knockout mice

Two doses of oxyntomodulin, low (30 nmol/kg) and high (3000 nmol/kg) were administered to GLP-1R knockout and wild type littermate controls. Similarly to study 4.3.1, the low dose of oxyntomodulin caused a reduction in blood glucose 15 minutes after administration to wild type mice (although this failed to reach statistical significance). However in GLP-1R knockout mice there was no reduction in blood glucose in response to low dose oxyntomodulin. High dose oxyntomodulin caused no statistically significant alteration in blood glucose in wild type or GLP-1R knockout mice, despite increasing plasma insulin levels in wild type mice. No effect of either dose of oxyntomodulin on insulin levels was seen in GLP-1R knockout mice. Together this data suggests that the GLP-1R is required for the hypoglycaemic effect of low dose oxyntomodulin, and for oxyntomodulin-induced insulin release. There was no significant increase in blood glucose in GLP-1R knockout mice in response to either dose of oxyntomodulin, thus glucagon receptor mediated effects could not be seen at the doses tested. This is in contrast to the results of a study published after the current studies had commenced, showing oxyntomodulin administration leads to reduced glucose tolerance compared to vehicle treatment under hyperglycemic clamp conditions (Du et al., 2012). However it is difficult to compare results under hyperglycemic clamp conditions to the results of the current studies.

4.4.2 Glucagon

4.4.2.1 The effects of glucagon on blood glucose and insulin release

The effects of a range of doses of glucagon on blood glucose and plasma insulin levels were tested for comparison with those of oxyntomodulin. It was expected that glucagon would cause a dose dependent rise in blood glucose. However, high doses of glucagon (100 nmol/kg and greater) did not appear to cause an increase in blood glucose compared to saline controls. Glucagon is known to have insulinotropic effects (Weir et al., 1989) and in the current study glucagon elevated plasma insulin in a dose dependent manner at doses of 100 nmol/kg and above. This might suggests that at
high doses the insulinotropic effects of glucagon dominate and thus blood glucose is not raised. Figure 4.10 shows a compilation of the glucose and insulin results from studies 4.3.2 and 4.3.3, and illustrates the difference in dose response between the hyperglycemic and insulinotropic effects of glucagon. It appears that peak glucose elevation occurs at low doses (10 nmol/kg) but insulin release does not occur to any measurable extent at this dose. It seems likely that at high doses (100 nmol/kg and greater) both effects occur, but the glucose release has already reached a maximum while insulin release continues to rise. This is supported by data from the measurement of liver glycogen, which although does not show a statistically significant effect of either dose of glucagon, appears to show that both 10 and 100 nmol/kg are capable of causing glycogen stores to become depleted to a similar extent.

The differing dose response relationships for the effects of on blood glucose and insulin release might suggest that the two responses are being mediated by different receptors. It is possible that the insulinotropic effects of glucagon are wholly or partly mediated by cross-reactivity with the GLP-1R. Data from in vitro studies suggests that glucagon is capable of cross-reacting with the GLP-1R to cause insulin release, and that both GLP-1 and glucagon receptors contribute to the insulinotropic effect (Moens et al., 1998; Moens et al., 2002). Thus I decided to test this hypothesis in vivo, in both a GLP-1R knockout mouse model, and using the GLP-1R antagonist EX 9-39.

**4.4.2.2 The effect of glucagon on blood glucose and insulin release in GLP-1R knockout mice**

A low dose of glucagon (10 nmol/kg) caused a rise in blood glucose in both wild type and GLP-1R knockout mice 15 minutes after administration. Thus the hyperglycaemic effect of glucagon is preserved in GLP-1R knockout mice. Wild type mice receiving a high dose of glucagon (100 nmol/kg), had significantly lower blood glucose than after receiving a low dose at this timepoint. In contrast the effect of this dose was significantly different in GLP-1R knockout mice, where a high dose of glucagon did increase blood glucose 15 minutes after administration compared to a saline control. This suggests the GLP-1R is necessary for the lack of hyperglycemia seen after high doses of glucagon.
Plasma insulin was significantly elevated after administration of 100 nmol/kg glucagon compared to saline controls in both wild type and GLP-1R knockout mice, although it appeared to slightly less elevated in GLP-1R knockout mice. This suggests that if cross reactivity with the GLP-1R is responsible for a portion of glucagon-induced insulin release, it is not wholly responsible and the difference was too small to be statistically significant. Overall the data on the effects of glucagon on blood glucose and insulin release suggest both high and low doses of glucagon are capable of causing mobilisation of stored glucose, but that high doses have a greater insulinotropic effect, perhaps partly mediated via the GLP-1R, and this may prevent any measurable rise in circulating glucose.

The results of this study may have been confounded by differences in baseline blood glucose between GLP-1R knockout and wild type mice and also between the saline and 100 nmol/kg glucagon wild type treatment groups. It will therefore be important to repeat this study in a larger number of animals, reducing the chance of significant baseline glucose variation between treatment groups, to confirm the results.

4.4.3 The effects of EX 9-39 on blood glucose and insulin release

In order to investigate the role of the GLP-1R in mediating the effects of glucagon and oxyntomodulin on blood glucose and insulin release without the confounding effects of altered glucose tolerance seen in GLP-1R knockout mice, I had hoped to block the GLP-1R using EX 9-39. However, interestingly, EX 9-39 itself had a hyperglycemic effect which has not been previously described. The hyperglycemic effect of EX 9-39 could be due to inhibition of basal insulin release, stimulation of glucagon release or an effect of its own on gluconeogenesis, glycogenolysis or glucose uptake.

EX 9-39 could affect insulin release via action at the GLP-1R. EX 9-39 has been shown to be an inverse agonist of the mouse GLP-1R in vitro (Serre et al., 1998) and thus could inhibit basal insulin release. However the hyperglycemic effect of EX 9-39 was preserved in GLP-1R knockout mice,
suggesting neither inverse agonism of the GLP-1R, nor a blockade of endogenous GLP-1 binding to GLP-1R, can be responsible for the observed effect. It also seems unlikely that cross reactivity with the GIP receptor could be responsible for the hyperglycemic effect. Although EX 9-39 is known to act as an antagonist at the GIP receptor at micromolar concentrations, antagonism of the GIP receptor appears to have only minor effects on glucose tolerance, and no effect on baseline glucose (Gremlich et al., 1995; Wheeler et al., 1995; Gault et al., 2003; Lewis et al., 2000). Furthermore no effect of EX 9-39 on insulin release was seen in the current studies suggesting antagonism of incretin receptors is unlikely to explain the hyperglycemic effect of EX 9-39.

There are no other receptors at which EX 9-39 is known to act as an agonist or antagonist. There have been several suggestions that there may be an, as yet unidentified ‘third’ receptor for the proglucagon derived peptides, largely because of the apparent divergent effects of oxyntomodulin and GLP-1 (Dakin et al., 2001; Baggio et al., 2004b). EX 9-39 has also been shown to block the cardioprotective effects of GLP-19-37 in mice, an effect which is preserved in GLP-1R knockout mice (Ban et al., 2010), and thus may represent an effect of EX 9-39 at an unknown receptor. Additionally both EX-4 and EX 9-39 have been shown to induce neurogenesis suggesting there may be a receptor at which they are both agonists or antagonists (Bertilsson et al., 2008). The effects of EX 9-39 seen on blood glucose in the current study cannot claim to be evidence of putative proglucagon related peptide receptor, but present an opportunity to investigate these effects further.

4.4.4 Discussion of methodologies used and their limitations

4.4.4.1 Investigations of glucose and insulin release in wild type mice

These studies were designed to be able to detect both hyperglycemic and hypoglycaemic effects of administered peptides. To detect a release of glucose glycogen stores must be intact, and thus the animals used were in the fed state. The major drawback of this method is the variability in basal blood glucose and insulin inherent to studies in ad libitum fed animals, meaning that larger numbers
of animals per group would have been desirable. The peptides investigated in these studies are also known to reduce intestinal motility (Patel et al., 1979; Pellissier et al., 2004), thus in ad libitum fed animals they may influence the levels of glucose entering the circulation from the gut.

Stress-induced elevation of glucose is a complication of most studies of rodent glucose homeostasis. Handling is associated with increased corticosterone and glucose in mice, and even mice handled regularly exhibit these responses (Balcombe et al., 2004). Glucose levels often appeared to rise in the saline control groups over the study period, which may render any elevation of blood glucose in response to glucagon or oxyntomodulin over and above this stress-induced rise more difficult to detect. It has been suggested that tail tipping should be carried out around 2 hours before sampling to ameliorate the effects of stress (Ayala et al., 2010), although in preliminary studies for this chapter this was often found to result in difficulty in sampling due to healing.

4.4.4.2 Investigations of glucose and insulin release in GLP-1R knockout mice

Baseline blood glucose levels often appeared higher in GLP-1R knockout mice, contradicting some previous reports. This may have reduced the apparent hyperglycemic effect of the peptides. In addition, GLP-1R knockout mice have altered responses to stress (MacLusky et al., 2000), and in the current studies the elevation of glucose seen in these animals after saline administration suggests they were subject to greater stress-induced glucose release than their wild type littermates. Furthermore GLP-1R knockout mice may be subject to developmental compensation and a number of confounding phenotypical differences to wild type mice as discussed in section 3.1.6.3.2.

4.4.5 Conclusions and Future Directions

The work in this chapter suggests that, as expected, oxyntomodulin can influence blood glucose and insulin through activation of the GLP-1R. The results also suggest that glucagon may have some effect on blood glucose and insulin release which are dependent on the presence of the GLP-1R. From the results of these studies I hypothesise that the effects of this GLP-1R action may mask the
hyperglycaemic effect of glucagon binding to its own receptor, and perhaps also of oxyntomodulin binding to the glucagon receptor.

The studies contained in this chapter are preliminary. Changes to blood glucose due to stress are apparent in saline control groups in most of these studies, particularly in GLP-1R knockout mice. Repeating these studies in surgically catheterised unrestrained animals may be important to remove the impact of stress on the responses seen. The effect of concurrent GLP-1 and glucagon receptor activation on blood glucose warrants further investigation, and an understanding of how these opposing effects are balanced is important for the design of co-agonists at these receptors for use as therapeutics. It may be useful to carry out isotopic tracer studies to determine how concurrent activation of these receptors affects the factors including tissue specific glucose uptake, glycogenolysis, and gluconeogenesis.

The hyperglycemic effect of EX 9-39 was unexpected and could be investigated further. Administration of EX 9-39 to a mouse model lacking both the GLP-1 and GIP receptors would be useful to determine whether EX 9-39 antagonism or inverse agonism of the GIP receptor is responsible for this effect. If the GIP receptor, like the GLP-1R, does not appear to be required to mediate the hyperglycaemic effect of EX 9-39, it may be helpful to carry out tracer studies after EX 9-39 administration to determine which aspects of glucose metabolism are affected by EX 9-39. Insulin release does not appear to be involved, and it may be useful to measure glucagon levels. Determining the mechanism of action of EX 9-39 would help to identify target tissues and receptors, and potentially to identify an unknown receptor which might mediate both this, and other published but unexpected effects of EX 9-39.
5 GENERAL DISCUSSION
The evolution of the preproglucagon gene has generated a family of peptides, all derived from the same sequence. The sequence for glucagon became duplicated and modified giving rise to two further peptides, GLP-1 and GLP-2 (Lopez et al., 1984). Differential processing of preproglucagon by prohormone convertases means two further hormones, oxyntomodulin and glicentin are produced, which contain the entire glucagon sequence (Rouille et al., 1994; Rouille et al., 1997). Over time the structure and functions of these peptides began to diverge. Divergence of function was dependant on the evolution of additional receptors. The glucagon, GLP-1 and GLP-2 receptors are all derived from a common ancestor and, similarly to the evolution of the peptides themselves, are the result of duplication of the original sequence (Irwin and Wong, 2005). Whilst most of the preproglucagon derived peptides will bind to all these receptors with low affinity, high affinity binding is usually only seen at one receptor. The exception to this is oxyntomodulin, which binds with moderate affinity to both the GLP-1 and glucagon receptors (Druce et al., 2009). The physiological roles of GLP-1 and glucagon are thought to have diverged significantly from one another in mammals, and as a co-agonist at both their receptors, oxyntomodulin may share effects with both peptides. This thesis aimed to examine the effect of activation of the GLP-1 and glucagon receptors on energy homeostasis and thus focused on the hormones glucagon, GLP-1 and oxyntomodulin.

The first experimental chapter of this thesis aimed to investigate the acute and chronic effects of glucagon and GLP-1R activation on food intake and body weight. It was found that both GLP-1 and glucagon inhibit food intake acutely at doses of a similar magnitude, with GLP-1 appearing approximately three times as potent as glucagon. When given in combination, doses of GLP-1 and glucagon too low to inhibit food intake, had an anorectic effect, which appeared to be at least additive. This somewhat mirrors the anorectic effect of oxyntomodulin, which is known to be of a similar potency to that of GLP-1 despite having close to a hundredfold lower affinity for the GLP-1R (Dakin et al., 2001), and suggested that glucagon receptor activation could contribute to the anorectic effect of co-agonists at the GLP-1 and glucagon receptors.
There has been great interest in the potential of GLP-1 and glucagon receptor co-agonists as treatments for obesity and type 2 diabetes (Pocai et al., 2009; Day et al., 2009). The combination of the anorectic effects of both hormones, increased energy expenditure by glucagon, and the stimulation of insulin secretion by GLP-1, would appear to be of therapeutic value. The evolutionary relationship between these peptides means development of a single molecule which can activate both receptors is feasible. However the hyperglycaemic effect of glucagon is a concern in the development of such an agent and thus in our laboratory it was decided to produce two separate agonists, which could be combined in any ratio, to achieve the best balance of effects on both weight loss and glucose tolerance.

Agents were designed to bind well to the human GLP-1 or glucagon receptor, but also to the rat and mouse receptors as these models would be used in the preclinical stages of development. They were also designed to be long-acting, a factor which limits the utility of the native peptides as therapeutics. When given in combination these agents reduced food intake, but also increased energy expenditure causing significant weight loss in both lean and obese rodent models. Furthermore chronic treatment with the peptides caused an improvement in glucose tolerance. The peptides used in these studies have since been developed further, to produce a second generation of peptides with enhanced pharmacokinetic profiles and efficacy. The ratio at which they will be administered will be determined empirically once they peptides reach phase II testing in obese diabetic humans.

The second experimental chapter of this thesis aimed to examine the anorectic effects of glucagon and GLP-1 further and to compare them to the dual agonist oxyntomodulin. c-fos immunohistochemistry was used to characterise for the first time the neuronal activation seen in appetite regulating centres in response to anorectic doses of glucagon. c-fos immunoreactivity was seen in the NTS, AP and CeA, and appeared to be dose-dependent. A similar pattern of immunoreactivity was seen in response to GLP-1. When compared directly at equivalently anorectic
doses GLP-1, glucagon and oxyntomodulin induced broadly similar patterns of c-fos expression in the brainstem and CeA. Oxyntomodulin appeared to induce higher levels of c-fos expression in the NTS and CeA than glucagon or GLP-1, and in the NTS most of the difference in c-fos expression appeared in the portion of the NTS immediately rostral to the obex and adjacent to the 4th ventricle. This may suggest that despite being an agonist at the GLP-1 and glucagon receptors, oxyntomodulin may act differently to either glucagon or GLP-1.

This is not the first report of differences in the action of GLP-1 and oxyntomodulin. Oxyntomodulin increases heart rate in mice, even in the absence of the GLP-1R (Sowden et al., 2007). Inhibition of pancreatic secretion is seen in response to physiological doses of oxyntomodulin, whereas the same doses of GLP-1 have no effect (Anini et al., 2000). Anorectic doses of oxyntomodulin restore fasting induced neuronal activation as measured by MEMRI in the ARC to the levels seen in ad libitum fed animals, whereas no effect of GLP-1 was seen despite being administered at the same dose, although this may be due to differences in pharmacokinetics or CNS penetration (Chaudhri et al., 2006). A further MEMRI study found that the effects of GLP-1 and oxyntomodulin on hypothalamic activation were readily distinguished, with oxyntomodulin appearing to affect activation in the ARC whereas GLP-1 affected activation in the PVN (Parkinson et al., 2009). All of these discrepancies could be explained by oxyntomodulin acting at the glucagon receptor in addition to the GLP-1R, although this hypothesis is yet to be tested.

With regards to the regulation of appetite however, the work of this thesis and of others (Baggio et al., 2004b), suggests that the GLP-1R is required for the action of oxyntomodulin. It is possible that differences in the effects of oxyntomodulin and GLP-1 occur downstream of GLP-1R activation. There has been some indication of signal bias downstream of the GLP-1R, with oxyntomodulin and GLP-1$^{36\cdot\text{NH2}}$ found to activate cAMP, ERK and Ca$^{2+}$ signalling pathways in different ratios (Koole et al., 2010). Signalling downstream of GLP-1R activation by oxyntomodulin has been shown to be modulated differently to that of GLP-1 in response to certain allosteric agonists (Koole et al., 2010;
Wootten et al., 2011). If oxyntomodulin and GLP-1 differentially activate intracellular signalling pathways in vivo this may explain the divergent effects on c-fos expression seen in experimental chapter two.

The increased c-fos expression seen in response to co-administered glucagon and GLP-1 presents another mechanism which might explain the greater c-fos induction seen in response to oxyntomodulin. It is possible that activation of two receptor systems simultaneously might lead to different effects than the activation of either individually. c-fos expression in response to oxyntomodulin in GLP-1R and glucagon receptor knockout mouse models, and in models of pharmacological blockade of these receptors would be required to investigate this further.

Experiments in the third chapter of this thesis suggest that EX 9-39, usually considered a specific GLP-1R antagonist, can elevate basal blood glucose in mice in the fed state even in the absence of the GLP-1R. EX 9-39 has been shown to antagonise the cardioprotective effects of GLP-19-37 in GLP-1R knockout mice (Ban et al., 2010) suggesting the existence of a receptor, likely closely related to the GLP-1R, at which EX 9-39 is also an antagonist. Such a receptor might also have an affinity for oxyntomodulin, and thus could be responsible for some of the divergent effects of GLP-1 and oxyntomodulin, although is unlikely to be capable of mediating oxyntomodulin’s anorectic effect. The clear effects of EX 9-39 on blood glucose seen in the experiments in this thesis may provide a useful tool by which to investigate the presence of this putative receptor.

The remainder of the third experimental chapter examined the effects of a range of doses of oxyntomodulin and glucagon on blood glucose and insulin release in fed mice. It was found that whilst, as expected, glucagon was capable of elevating blood glucose, that at higher doses of glucagon this effect was not present. However, in GLP-1R knockout mice, glucagon had hyperglycemic effects at both low and high doses. Thus it appears that at higher doses glucagon is capable of activating the GLP-1R, and may manifest in reduced insulin release in GLP-1R knockout mice in response to high dose glucagon.
Activation of the GLP-1R by glucagon also appears to be responsible for glucagon-induced anorexia as seen in experimental chapter 2. Whilst cross reactivity of glucagon with the GLP-1R has been seen in vitro, in both this thesis and by other investigators (Thorens, 1992; Moens et al., 1998), the affinity of glucagon for the GLP-1R is three orders of magnitude lower than that of GLP-1. I found that to inhibit feeding to a similar extent, glucagon had to be administered at doses approximately three times higher than GLP-1. In experiments measuring blood glucose the dose of glucagon which maximally increased blood glucose was only an order of magnitude lower than one which appeared not to raise blood glucose due to cross-reaction with the GLP-1R. Thus in vivo it appears that GLP-1R activation may be more relevant to the effects of glucagon than in vitro studies would suggest.

A similar conclusion might also be made for oxyntomodulin which induced the expected, GLP-1R dependent, hyperglycemic effect at lower doses, but at high doses failed to reduce blood glucose despite increasing insulin release. This might be due to activation of the glucagon receptor and thus inducing glucose release which counteracts the effect of increased insulin secretion. Investigations of the effects of oxyntomodulin in the glucagon receptor knockout mouse could be carried out to test this hypothesis.

Whilst the high doses administered in these studies cannot claim to be physiological, the studies contained in this thesis suggest that rather than glucagon, GLP-1 and oxyntomodulin being agonists at the glucagon, GLP-1 and both the glucagon and GLP-1 receptors respectively, that perhaps all three hormones are co-agonists, but with differing ratios of affinity. It would be interesting to repeat some of the blood glucose studies carried out in this thesis with GLP-1, and with the glucagon receptor knockout mouse as an additional tool, to determine whether, like oxyntomodulin and glucagon, there may be a dose at which activation of both receptors appears relevant.

These pharmacological effects are of relevance to the development of analogues of these peptides as therapeutics. It may be that the effects we expect the three hormones to have based on physiological studies, will not be reflected in the effects of analogues given at therapeutic doses.
Thus, with respect to glucose homeostasis in particular, the design of a peptide or peptides with affinities which in vitro, appear favourable for GLP-1R mediated insulin release without a significant concurrent glucagon receptor mediated hyperglycemic effect, might be misleading.

The experiments within this thesis have compared the effects of glucagon, GLP-1 and oxyntomodulin on feeding, c-fos expression in appetite regulating centres, and on blood glucose and insulin release. Overall I have found more similarities than differences between these structurally and evolutionarily related peptides. They are all anorectic. They all induce similar patterns of c-fos expression in the brainstem and amygdala, and these could not be differentiated by immunohistochemical characterisation of the markers examined in this thesis. The effects of glucagon and oxyntomodulin on glucose homeostasis, whilst at some doses opposed one another, at others were very similar, suggesting both have the potential to be co-agonists at the GLP-1 and glucagon receptors. This degree of similarity is not surprising given the common ancestral origins of these peptides. It has been suggested that prior to the divergence of fish and mammals, both GLP-1 and glucagon had similar functions, and both bound to the glucagon, GLP-1 and possibly GIP receptors present at that time (Irwin and Wong, 2005). It was not until after fish and mammals diverged that GLP-1 gained its incretin function, and in fish it remains a major glycogenolytic hormone.

The co-existence of anorectic, energy expenditure and insulinoactive effects in one family of peptides is fortuitous for the development of therapeutics for obese type 2 diabetics, and oxyntomodulin serves as an example of the potential for these beneficial effects to be delivered by one molecule. However the experiments in this thesis show that there are still many aspects of the mechanism of action of these peptides that are still not understood. Future investigations of the central mechanisms of the effects of PPG derived peptides on feeding can build on the identification of POMC and catecholaminergic neuronal activation by these peptides in the NTS. The cross-reactivity of glucagon with the GLP-1R, which may be relevant to the effects of glucagon on feeding and glucose homeostasis in vivo, could be examined further and may be relevant to the perceived
mismatch in GLP-1R affinity and in vivo efficacy of oxyntomodulin. Finally the experiments using EX 9-39 suggest the search for another receptor in the glucagon-receptor family should continue and may open up new avenues of investigation for the effects of the preproglucagon derived peptides.
6 APPENDICES
6.1 **APPENDIX ONE: RADIOIMMUNOASSAYS**

Radioimmunoassays (RIAs) used were derived and maintained by Professor MA Ghatei (Professor of Regulatory Peptides, Metabolic Medicine, Faculty of Medicine, Imperial College). All reagents and materials other than peptides were supplied by Sigma.

The principle of RIA is the competition between a radioactive and non-radioactive antigen for a fixed number of antibody binding sites. When the unlabelled antigen in standards or samples and a fixed amount of labelled antigen are allowed to react with a constant and limiting amount of antibody, decreasing amounts of labelled antigen are bound to the antibody as the amount of unlabelled antigen is increased. The RIA is incubated and allowed to reach equilibrium, according to the equation:

\[ \text{Ag} + \text{Ab} + \text{Ag} \rightarrow \text{AgAb} + \text{AgAb} \]

Ag = unlabelled antigen

*Ag = radiolabelled antigen

Ab = antibody

Separation of the free radiolabelled antigen from the antibody complex is achieved by adsorption of the free radiolabelled antigen. Dextran is added to a charcoal suspension to block the larger holes in the porous charcoal. The suspension is then added to the RIA tubes, where it traps the free radiolabelled antigen. The tubes are then centrifuged and the supernatant (bound antibody complex) and carbon pellet (free radiolabelled antigen) separated by aspiration, and counted in a gamma counter (NE 1600, NE Technology Ltd.). The data are used to construct a standard curve from which the values of the unknowns can be obtained by interpolation.
Inter-assay variation can be calculated by assaying aliquots of the same sample in each assay performed and comparing the concentrations obtained in each. To measure and correct for baseline drift, tubes with no sample (‘zero’ tubes) are placed at regular intervals throughout the assay and standard curves are performed at the beginning and end of each assay.

The following tubes are important for the assessment of the quality for the label, antibody and overall performance of the assay:

**Blank:** contains buffer and label. Assesses non-specific binding of the label and label integrity.

½ X: contains buffer, antibody and half the usual concentration of label. Assesses if greater sensitivity could be achieved by adding half the volume of label.

2 X: contains buffer, antibody and twice the usual concentration of label. Assesses if greater sensitivity could be achieved by adding double the volume of label. Also used to assess label integrity

Zero tubes: contains buffer, label and antibody. allows assessment of assay drift.

Excess antibody: contains label and antibody. assesses the immunological integrity of the labelled peptide.

Quality Controls: includes buffer, label antibody and previously aliquoted samples containing high and low levels of the antigen. These tubes allow the assays to be standardised.
6.2 Appendix Two: The effect of GCGAg on acute food intake in rats

Figure 6.1: Effect of GCGAg on food intake over 24 hours in rats. Rats were fasted overnight, injected at the onset of the light phase subcutaneously with either saline or GCGAg (500 nmol/kg) and given a measured amount of food. Food was reweighed at the specified times. A: cumulative food intake, B-F: interval food intake N=6. *p<0.05, **p<0.01, ***p<0.001
6.3 Appendix Three: The Effect of GLP-1 on Food Intake in the Presence and Absence of Ex 9-39

Figure 6.2: Effect of GLP-1 on food intake in the presence and absence of Ex 9-39. 0-30 minute food intake (Ex 9-39 or saline administered at t=-5, GLP-1 or saline administered at t=0. Ex 9-39 25 μmol/kg, GLP-1 300 nmol/kg) Mice were fasted overnight, injected at the onset of the light phase s/c and given a measured amount of food. Food was reweighed at the specified times. N=7 *p<0.05, ** p<0.01 ***p<0.001.
6.4 **APPENDIX FOUR: COORDINATES OF SECTIONS USED FOR C-FOS IMMUNOHISTOCHEMISTRY**

<table>
<thead>
<tr>
<th>Nucleus/Region of interest</th>
<th>Coordinates (mm relative to Bregma)</th>
<th>Number of sections Glucagon study</th>
<th>Number of sections GLP-1 study</th>
<th>Number of sections combination study</th>
<th>Number of sections GLP-1, glucagon, OXM comparison study</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP</td>
<td>-7.32 to -7.64</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>cNTS</td>
<td>-7.08 to -7.92</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>IPBN</td>
<td>-5.20 to -6.68</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>ARC</td>
<td>-1.22 to -2.70</td>
<td>5</td>
<td>4</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>PVN</td>
<td>-0.58 to -1.22</td>
<td>3</td>
<td>3</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>VMH</td>
<td>-1.06 to -2.06</td>
<td>2</td>
<td>3</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>DMH</td>
<td>-1.34 to -2.06</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>CeA</td>
<td>-0.82 to -1.82</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 6.1: Coordinates of sections used for c-fos immunohistochemistry
6.5 APPENDIX FIVE: ACTH IMMUNOREACTIVITY IN THE HYPOTHALAMUS

Figure 6.3: ACTH immunoreactivity in the hypothalamus. Representative sections of ACTH immunoreactivity in the mouse hypothalamus. A 40x magnification B 100x magnification.
6.6 **APPENDIX SIX: SOLUTIONS**

**Fixative**

15g of NaCl, 28.2g Na2HPO4.2H2O and 5.44g KH2PO4 were dissolved in 800mls H2O. 100 ml of 40% formaldehyde were added and the solution made up to 1L. Adjust to pH 7.4 with NaOH.

**Flush**

15g of NaCl, 28.2g Na2HPO4.2H2O and 5.44g KH2PO4 were dissolved in 900mls H2O and the solution made up to 1L. Adjust to pH 7.4 with NaOH.

**1% Copper sulphate solution**

Dissolve 1 g of copper sulphate and 0.9 g NaCl in 100 ml ultra pure water.

**Dextran coated charcoal**

Add 2.4g charcoal and 0.24g dextran to 100ml phosphate buffer with gelatine and mix for 20 mins at 20°C

**DNA loading buffer**

Mix 3.125ml 80% glycerol, 50μl 0.5M CH14H2O8Na2.2H2O (EDTA) and 6.075ml ultra pure water, add 10mg orange G.

**0.5M ethylenediaminetetra-acetic acid (EDTA)**

Dissolve 186.1g CH14H2O8Na2.2H2O (EDTA) in 800ml ultra pure water and adjust pH to 8.0.

Make up to 1L with ultra pure water.

**GLP-1R RBA buffer**

0.02M HEPES pH 7.4, 5mM CaCl2.2H2O (Sigma), 1mM MgCl2.8H2O (Sigma), 1% BSA, 0.1 mM diprotin A, phenylmethanesulfonylfluoride (PMSF) (dissolved in 100% ethanol, 0.2mM) and 0.025%Tween.

**Glucagon Receptor RBA buffer**

0.025M HEPES pH 7.4, 2mM MgCl2.8H2O (Sigma) 1% BSA, 0.1 mM diprotin A, PMSF (dissolved in 100% ethanol, 0.2mM) 0.05%Tween.

**Homogenation Buffer**
50mM HEPES pH 7.4, 0.25M sucrose, 10μg/ml soybean trypsin inhibitor (Sigma-Aldrich), 0.5μg/ml pepstatin (Sigma-Aldrich), 0.5μg/ml antipain (Sigma-Aldrich), 0.5μg/ml leupeptin (Sigma-Aldrich), 0.1mg/ml benzamidine (Sigma-Aldrich) and 1ml 100KIU/ml aprotinin (Trasylol, Bayer)

1M magnesium chloride

Dissolve 203.3g MgCl2 in 1L ultra pure water.

Phosphate buffer (0.06M) (RIA buffer)

Dissolve 48 g of Na2HPO4.2H2O, 4.13 g KHPO4, 18.61 g C10H14H2O8Na2.2H2O, 2.5 g NaN3 in 5L of ultra pure water that has been boiled and allowed to cool. Measure the pH to confirm it is 7.4±0.1. Store at 4°C.

Phosphate buffer (0.06M) with gelatin

The buffer is produced as above with 12.5 g of gelatine dissolved in the boiling ultra pure water then cooled before the addition of the other ingredients.

10x Phosphate buffered saline (PBS)

Add 80g of NaCl, 2g KCl, 14.4g Na2HPO4, 2.4g KH2PO4 to 800ml ultra pure water. Adjust volume to 1L with ultra pure water, adjust pH to 7.4 and sterilise by autoclaving.

1x PBS

Add 100ml 10x PBS to 900ml ultra pure water. Sterilise by autoclaving.

10M sodium hydroxide

Dissolve 400g of NaOH in 500ml ultra pure water. Once dissolved, make up to 1L with ultra pure water.

30% sucrose solution

Dissolve 300g of sucrose in 1L 1xPBS.

50 x Tris-acetate-EDTA (TAE) buffer

Dissolve 242g Trizma base in 843ml ultra pure water and mix in 57ml glacial acetic acid and 100ml 0.5M C10H14H2O8Na2.2H2O.

TAE running buffer

To 1L ultra pure water, add 75μl ethidium bromide and 10ml 50 x TAE.


Ref Type: Generic


Food and Drug Administration. FDA approves weight-management drug Qsymia. FDA NEWS RELEASE. 17-7-2012a.

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281